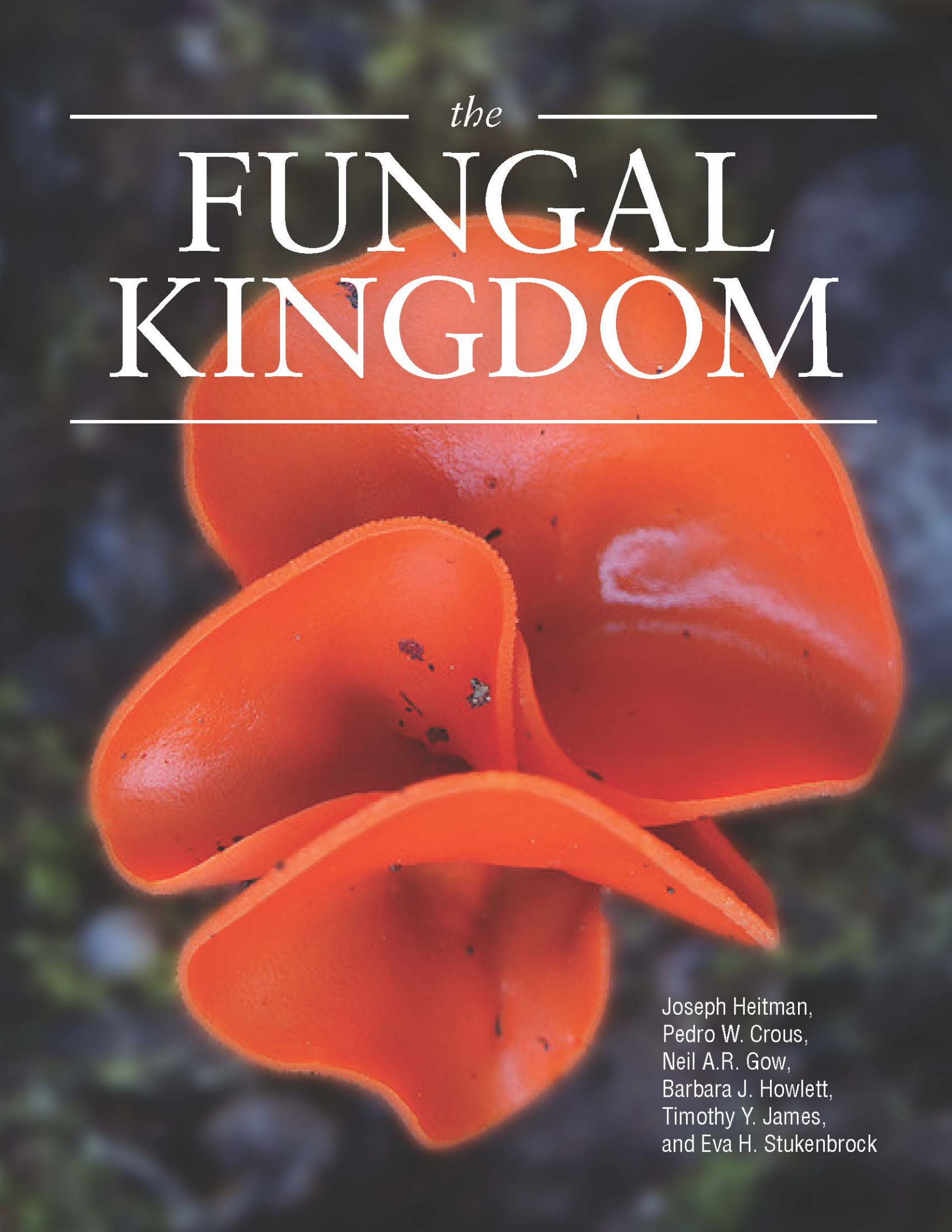


the FUNGAL KINGDOM

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Pedro W. Crous,
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Foreword

Studies attempting to estimate fungal biodiversity mainly serve to underscore the limitations of our current awareness of the likely total number of species—currently estimated to be around 5 million. As environmental organisms, fungi influence the global ecology and the recycling of nutrients in the biosphere and also, both positively and negatively, the viability of many plants and animals. As simple eukaryotes that can be facilely studied and experimentally manipulated, fungi serve as important models that have profoundly influenced our understanding of life by enabling the identification and analysis of conserved mechanisms underpinning the growth, cell division, and death of all eukaryotic cells.

Fungal research is a vibrant and exciting area, but it is often dispersed among distinct scientific communities with different cultures and traditions. The objective of bringing together this broad research portfolio into a book and collection of contemporary reviews is therefore a useful, important, but challenging objective. This ASM-sponsored volume is unusual in uniting a community of mycologists and cell biologists with the common objective of illustrating the state of our understanding of both model fungi and organisms with specific environmental, pathogenic, or biological relevance. The book includes chapters where the focus is at the molecular, cellular, or organismal levels of spatial organization and includes fungi from all the major phylogenetic groupings. The result is an anthology of articles that defines the current trajectory of current research and questions for the next generation of investigators. The authors are leaders in their respective fields, and the editorial style is such that the work has achieved an overview of the field as a whole in a form that is useful both for the specialist and for those seeking understanding in areas in which they may be unfamiliar.

Some of the most profoundly important questions in biology have been explored using fungi, yeasts, and molds—and it is likely that this paradigm will continue into the future. This book should serve to stimulate a new generation of mycologically inclined scientists to investigate the extraordinary diversity and experimental accessibility of members of the mycobiota.

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Preface

Our fascination with the Fungal Kingdom is a natural and ancient one and based on (i) the roles of fungi in the production of a variety of foods and beverages, and even as a source of food themselves; (ii) their global ecological impact, especially as the cause of devastating infections of humans and other animals and of plants, including many crops grown around the world; and (iii) their roles as fundamental model systems in genetics and biological research.

The earliest fascination with fungi likely began with humans across the globe foraging for food sources, often in the context of forests, and the prominent role of mushroom fruiting bodies associated with trees, either as mycorrhizal species on roots of living trees or as wood-degrading fungi on dead plant materials. These mushroom species include popular and delicious edible ones such as porcini, chanterelles, and truffles as well as shiitake, oyster, portobello, and more. In aggregate, the global commercial mushroom market was ~$35 billion US in 2015 and is anticipated to grow to as much as ~$60 billion by 2021. The species named are representatives of just one phylum (the Basidiomycota) within the broader Fungal Kingdom, which is now estimated to include as many as 2.2 to 3.8 million or more distinct species and at least seven phyla (Ascomycota, Basidiomycota, Mucoromycota, Zoopagomycota, Blastocladiomycota, Chytridiomycota, and the Cryptomycota/Rozellomycota).

Interest in fungi also derives from their key roles in the production of other foods and beverages, including the prominent role of the yeast *Saccharomyces cerevisiae* (Ascomycota) in production of beer, wine, Champagne, and bread. Yet other fungi such as *Penicillium chrysogenum* produce natural products like the antibiotic penicillin, which revolutionized medicine and health care and contributed to dramatically prolong the human lifespan by enabling effective treatment of infectious diseases. Finally, pathogenic fungi cause (i) devastating, life-threatening systemic infections of humans and also other diseases including allergies, blindness, vaginitis, and common skin infections including dandruff and athlete's foot; (ii) widespread infections of animals including the ones that are devastating bat populations in North America and causing extinctions in frog species around the world; and (iii) the majority of infections that occur in plants, including many crop species, which lead to famines and disruptions of food supply with billions of dollars lost in agriculture annually. Whatever your vantage point, the impact of fungi on the biology of our planet, the development of human civilization, and our daily lives and health is writ large.

A few decades ago the position of the Fungal Kingdom within the broader eukaryotic tree of life was unclear, and in many instances it was thought and even taught that the fungi were more closely related to plants than to animals. The overt morphological features of mushrooms likely contributed to this view, along with uncertainty about where to place unicellular microbes within the context of complex multicellular organisms such as animals. But with the advent of molecular phylogenetic studies, the placement of fungi within the eukaryotic tree of life came into sharper focus, and we now appreciate that fungi are much more closely related to animals than they are to plants. This evolutionary kinship is in fact so certain that the Animal Kingdom and the Fungal Kingdom are now appreciated to be sister groups within the broader Opisthokonta supergroup of eukaryotes. This revised phylogenetic placement reveals much about the con-

servation of molecular mechanisms of life and contributes to making fungi exceptional models to understand the form and function of other eukaryotes, including the Animal Kingdom.

In concert with these advances in molecular phylogenetics and taxonomy, two other fields have revolutionized our understanding of the Fungal Kingdom. First, advances in genetics of fungi have contributed to illuminate their unique and conserved biological properties. Studies on fungi have contributed profound insights as models for all of biology, such as the discovery of RNA interference (RNAi) pathways and how they operate to silence transgenes and protect genome integrity; the detailed mechanisms and operation of a biological clock and the key concept that this involves a molecular oscillator; the first experimental demonstration of DNA sequences that can function as telomeres, centromeres, and eukaryotic origins of replication; the discovery of the first components of the nuclear pore; the description of the secretory pathway and discovery of autophagy; the elucidation of how the cell cycle is orchestrated; the understanding of the impact of both ploidy and aneuploidy on cell functions; insights into how species evolve and species boundaries operate; and the discovery that the mismatch repair system is required for the stability of DNA repeats, leading to insights into how similar mutations lead to colon cancer in humans. Taken together, genetic, genomic, and cell biological studies have brought representatives of the entire Fungal Kingdom of life into focus and thereby made an invaluable contribution to our understanding of how eukaryotic organisms evolved and function. Indeed, seven Nobel prizes have been awarded to scientists studying yeasts and molds as model organisms that explain fundamental aspects of cell biology. These were awarded to Alexander Fleming, Ernst Chain, and Howard Florey in 1945 for the discovery of penicillin by *Penicillium notatum*; to George Beadle and Edward Tatum in 1958 for their One Gene = One Enzyme hypothesis in *Neurospora crassa*; to Paul Nurse and Leland Hartwell in 2001 for cell division and cancer in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*; to Roger Kornberg in 2006 for eukaryotic gene transcription in *S. cerevisiae*; to Jack W. Szostak (shared) in 2009 for chromosome telomeres in *S. cerevisiae*; to Randy Schekman in 2013 (shared) for machinery regulating vesicle traffic in *S. cerevisiae*; and to Yoshinori Ohsumi in 2016 for autophagy in S. cerevisiae.

In concert with advances in genetics, subsequent advances in genome sciences have provided the complete genome sequences for an increasing number of fungal species, now >1,000, and in some species a staggering number of individual representative genomes (500 or more). In fact, a fungal species was the first eukaryotic organism to have its genome completely sequenced (the model budding yeast S. cerevisiae). Second, advances in genetics and cell biology have contributed to provide a detailed view of how the genome contributes to the functions of the cell and of the organism. Together, these advances in genomics and genetics provide a "blueprint" for how these species operate and have evolved at a cellular level, and consequently they offer a wealth of knowledge about how representative species in the fungal kingdom function and the diversity that lies within. This diversity spans from the most basic way that a fungal cell is organized, either as a yeast or as a filamentous hypha, to the myriad ways these species interact with their environment, from the aquatic basal fungi (Chytridiomycota, Cryptomycota), to fungi that are associated with plants and were critical for their emergence from the oceans and colonization of the planet, to fungi that are pathogens of plants or animals. This diversity also extends to the biological behavior and cell biology of fungi, including for example fungi that can sense light and those that have evolved to be insensitive to light (blind), the modes of sexual reproduction including heterothallism and homothallism, the loss and retention of RNAi pathways, the replacement of regional centromeres by point centromeres, and the retention of flagella in basal fungi versus their loss in fungal branches that evolved the ability to be aerially dispersed.

Given the rapidly advancing fields of fungal genetics and genomics, and mycology more generally, we increasingly found ourselves in need of a compendium to organize this information and to serve as a reference to guide both our own efforts and those of others whose research focuses on or interfaces with fungi. We have assembled a team of six editors with complementary and diverse interests and enlisted a cadre of 170 experts in the field who as authors have contributed the 54 chapters that comprise *The Fungal Kingdom*. We have organized the book into nine different sections to present related material together and provide a framework for organization. Each chapter is designed to be self-contained, such that any reader may choose to read any given chapter in isolation or a series of related chapters from one section. At the same time, the book has a coherent theme of focusing on the diversity, importance, impact, dangers, and beauty of the fungi and could therefore be read as a continuous text. As modes of publication have advanced, this book is also an experiment in that it is available as a hard copy printed volume, as an electronic book, and as individual chapters available electronically or in their published form as part of the *Microbiology Spectrum* journal from ASM Press.

It is our hope, and our goal and intention, that this book both takes stock of the current state of knowledge in the field and also spurs further investigations into topics of interest that stem from the information contained herein. We invite you to peruse the current state of knowledge here and hope these musings spur you to join us in further advancing the field. We also invite you to communicate to us your experiences with the book. It is our fervent hope that advances over the next several decades will ultimately render this book out of date, and therefore in need of revision or replacement, as the field advances.

In closing, we would like to thank the numerous individuals who have contributed to advance our understanding of the Fungal Kingdom and, by extension, to the stimulation and realization of this text. We wish to dedicate this effort to the scientific mentors who trained and inspired us, and also to our significant others, children, and families, without whose forbearance and tolerance this effort would not have been possible. Finally, we thank our tireless ASM editors, Greg Payne, Lauren Luethy, and Ellie Tupper, who with administrative assistance from Melissa Palmer made this text possible through their indefatigable and enthusiastic efforts.

Joseph Heitman
Barbara J. Howlett
Pedro W. Crous
Eva H. Stukenbrock
Timothy Y. James
Neil A. R. Gow

Editors



Joseph Heitman, M.D., Ph.D., is James B. Duke Professor and Chair, Department of Molecular Genetics and Microbiology, Duke University. His research focuses on model and pathogenic fungi, including mating-type locus evolution, transitions in sexual reproduction modes, fungal virulence, and genome evolution. His work discovered FKBP12 and TOR as the targets of rapamycin and unisexual reproduction. He is fellow of the American Society for Clinical Investigation, American Academy of Microbiology, AAAS, and Association of American Physicians and received Burroughs Wellcome, AMGEN, Squibb, and NIH MERIT awards. He is an editor or board member for numerous journals and has edited seven textbooks.



Barbara Howlett, Ph.D., is an Honorary Professor in the School of BioSciences, the University of Melbourne. She studies blackleg, a fungal disease of canola. She has exploited a "genome to paddock" approach: identifying fungal genes and genetic mechanisms crucial for disease, and developing disease management strategies that are now implemented by Australian canola growers. She also has discovered biosynthetic pathways for fungal toxins involved in diseases of both plants and animals. Howlett is a Fellow of the American Academy of Microbiology, the Australian Academy of Science, and the Australasian Plant Pathology Society and an Honorary Member of the American Mycological Society.



Pedro W. Crous, Ph.D., D.Sc., is professor in evolutionary phytopathology at the Universities Wageningen and Utrecht, the Netherlands, where he is presently the Director of the Westerdijk Fungal Biodiversity Institute. His main research interests are fungal systematics and the characterization of fungal plant pathogens. To coordinate global research on fungal biodiversity, he launched MycoBank, is the author of several thousand fungal taxa, and is a key role player in DNA barcoding of fungi. He is an editor or board member for numerous journals and has authored or edited several textbooks.



Eva H. Stukenbrock, Ph.D., is a Max Planck professor at the Christian-Albrechts University of Kiel and the Max Planck Institute for Evolutionary Biology, Plön, Germany. Her research focuses on population biology and evolution of plant-associated fungi. Work in her group integrates evolutionary genomics with experimental and molecular approaches. She received her Ph.D. from the ETH Zurich, Switzerland, and established her independent line of research at Aarhus University, Denmark. Since 2010 she has been affiliated with the Max Planck Society in Germany, first as a research group leader and since 2014 as Max Planck fellow.



Timothy Yong James, Ph.D., is an Associate Professor in the Department of Ecology and Evolutionary Biology at the University of Michigan and the Lewis E. Wehmeyer and Elaine Prince Wehmeyer Chair in Fungal Taxonomy. His research interests include resolving the fungal tree of life and the structure of genetic variation within genomes, individuals, and populations. His research organisms span the fungal tree, with emphasis on the zoosporic fungi or chytrids. He is an Associate Editor of the journal *Mycologia* and recipient of the Alexopoulos Prize from the Mycological Society of America.



Professor Neil A. R. Gow, Ph.D., has research interests in medical mycology and in particular the structure and function of the fungal cell wall in relation to host-pathogen interactions. He is a founding member of the Aberdeen Fungal Group (AFG), which was established as the MRC Centre for Medical Mycology at the University of Aberdeen, United Kingdom. He has served as president of the British Mycological Society, the International Society for Human and Animal Mycology, and the Microbiology Society and has been elected as a FAAM, FRS, FRSE, and FMedSci.

Fungal Branches on the Eukaryotic Tree of Life

I

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The Fungal Tree of Life: From Molecular Systematics to Genome-Scale Phylogenies

1

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INTRODUCTION

In 1996 the genome of *Saccharomyces cerevisiae* was published and marked the beginning of a new era in fungal biology (1). Since then, rapid advancements in both sequencing technologies and computational biology have resulted in the sequencing of genomes for more than 800 species (e.g., http://genome.jgi.doe.gov/fungi/). These genomes represent a windfall of data that are informing evolutionary studies of fungi and the search for biological solutions to alternative fuels, bioremediation, carbon sequestration, and sustainable agriculture and forestry (2). Indeed, the marriage between genomics and phylogenetics occurred early, both in the use of phylogenetic techniques to study genome evolution and in the use of genome-scale data to infer evolutionary relationships. In this article we will review the impact of genomic-scale phylogenies, along with standard molecular phylogenies, on our understanding of the evolution of the fungal tree of life and the classification that communicates it.

Genomic data provide the maximum amount of discrete genetic information available for phylogenetic analyses, and hundreds to thousands of genes have been identified as useful phylogenetic markers (3). Markov clustering algorithms have been proven powerful tools to identify orthologous clusters of proteins that can be filtered for single-copy clusters that are useful in phylogenetic analyses (4). This approach has transformed phylogenetics by no longer requiring selection of an *a priori* set of markers (e.g., rDNA, RPB2, etc.), but rather promotes the mining of a data set of genomes for the largest set of appropriate markers. Furthermore, hidden Markov models have proven to be valuable tools for identifying and retrieving these markers in newly sequenced genomes and rapidly growing genome-scale phylogenetic data sets (5).

The estimation of species trees from genome-scale data sets is not without challenges, however. Phylogenetic analyses of genomic data have revealed that different genes within a genome can have different evolutionary histories, i.e., phylogenetic conflict (6). Sources of conflict include incomplete lineage sorting (or deep coalescence), hybridization, and horizontal gene transfer, and the detection and characterization of this conflict in the context of phylogenetic inference are still in their infancy (7). The application of standard measures of topological support, such as the bootstrap partition, can also be difficult to interpret, due to the observation that nodes that resolve differently in different gene data sets can have high or maximum bootstrap partition values in a subset of analyses (e.g., 8, 9). At the time of the writing of this manuscript the majority of genome-scale phylogenetic analyses focus on the analysis of concatenated superalignments, but development and use of supertree methods, gene tree-species tree reconciliations, and alternative measures of nodal sup-

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port are increasing (e.g., 8, 10) and will be developed further over the coming years.

Despite the challenges mentioned above, phylogenetic analyses of genome-scale data sets, and more traditional multigene data sets, have greatly advanced our understanding of fungal evolution. Historically, the fungi were divided into more or less four groups—chytridiomycetes, zygomycetes, ascomycetes, and basidiomycetes—defined by morphological traits associated with reproduction. (Note: The suffix "-mycetes" is used to denote a class-level taxonomic group in fungal nomenclature, e.g., Agaricomycetes. Its use as a lowercase noun, however, signifies an informal name and not an explicit taxonomic rank.) The chytridiomycetes, or zoosporic fungi, were recognized based on their production of zoospores, characterized by a single posterior, smooth flagellum. The zygomycetes were characterized by gametangial conjugation and the production of zygospores, coenocytic hyphae, and typically asexual reproduction by sporangia. The ascomycetes and basidiomycetes were identified by the production of asci and basidia, respectively, possession of regularly septate hyphae, and a dikaryotic nuclear phase in their life cycle. The classification of the kingdom Fungi used here recognizes eight phyla (Fig. 1, Table 1), with the zoosporic fungi comprising the first three lineages of the kingdom—Cryptomycota/Microsporidia, Chytridiomycota, and Blastocladiomycota—since the divergence from the last universal common ancestor (LUCA) of Fungi.

The resolution of zoosporic fungi as paraphyletic rejects the flagellum as a diagnostic trait (synapomorphy) for a monophyletic group of flagellated fungi. Rather, it is an ancestral (symplesiomorphic) trait inherited from the LUCA of the kingdom Fungi. Most extant species of fungi are nonflagellated and are the result of multiple losses of the flagellum during fungal evolution. Two losses of the flagellum have occurred, giving rise to the Microsporidia and the most recent common ancestor (MRCA) of the remaining phyla of zygomycetes, ascomycetes, and basidiomycetes. Inferences of additional losses of the flagellum are required for the placement of nonflagellated species among the Chytridiomycota (11) and possibly for the placement of the enigmatic zoosporic genus *Olpidium* among zygomycetes (12), but the absolute number of losses is unclear. The zygomycetes are also paraphyletic and are classified in two phyla: Zoopagomycota and Mucoromycota (13). This classification rejects the zygospore as a synapomorphy for the zygomycetes; rather, it was inherited from the MRCA of terrestrial fungi and lost in the MRCA of ascomycetes and basidiomycetes. The monophyly of ascomycetes and basidiomycetes has been confirmed, and they are classified as the phyla Ascomycota and Basidiomycota, respectively, of the subkingdom Dikarya (14). More information on character evolution will be highlighted throughout this article.

One of the greatest challenges in evolutionary biology of fungi is accurately estimating geologic dates of the origin of the kingdom Fungi, emergence of the major phyla, and diversification of extant lineages (15). Our knowledge of the fossil record of fungi is less than that of plants and animals, but there does exist an increasing number of fossils that can be assigned to major groups of fungi based on their morphological similarity to extant taxa (16). An important observation is that morphologies associated with Blastocladiomycota, Chytridiomycota, Mucoromycota, and Ascomycota are present in the early Devonian and are associated with the earliest known land plant flora of the Rhynie chert. This observation, in combination with relaxed molecular clock analyses (e.g., 17, 18), suggests that the common ancestors of the phyla of Fungi are in fact old and may have been among the first terrestrial organisms. The interpretation of fungus-like fossils can be challenging, however, as it is difficult to interpret some morphologies that are not present among extant lineages as definitive representatives of the kingdom Fungi (19).

In this article we will highlight the major phyla of fungi based on the current understanding of the fungal tree of life. In doing so, we will outline their phylogenetic diversity and classification, provide examples of important taxa for each group, and discuss advancements in our understanding of morphological and ecological evolution through the analysis of genomic and molecular data. There are many specialized terms used in this article, and we are unable to fully define all of them here. However, Fig. 2 to 5 provide examples of taxa and morphologies discussed herein, but the reader is directed to more traditional textbooks in mycology for more detailed discussions.

ZOOSPORIC FUNGI (Fig. 2)

Before we consider zoosporic fungi, a brief discussion of some unique aspects of their development and morphology is necessary. The morphology of zoosporic fungi varies depending on the extent of their thallus development, number and position of reproductive structures, and position in the substrate. Thalli may exist as single cells with sparse rhizoidal systems to more extensive networks of rhizoids (rhizomycelia) and mycelial thalli. Endobiotic chytrids are partially or completely

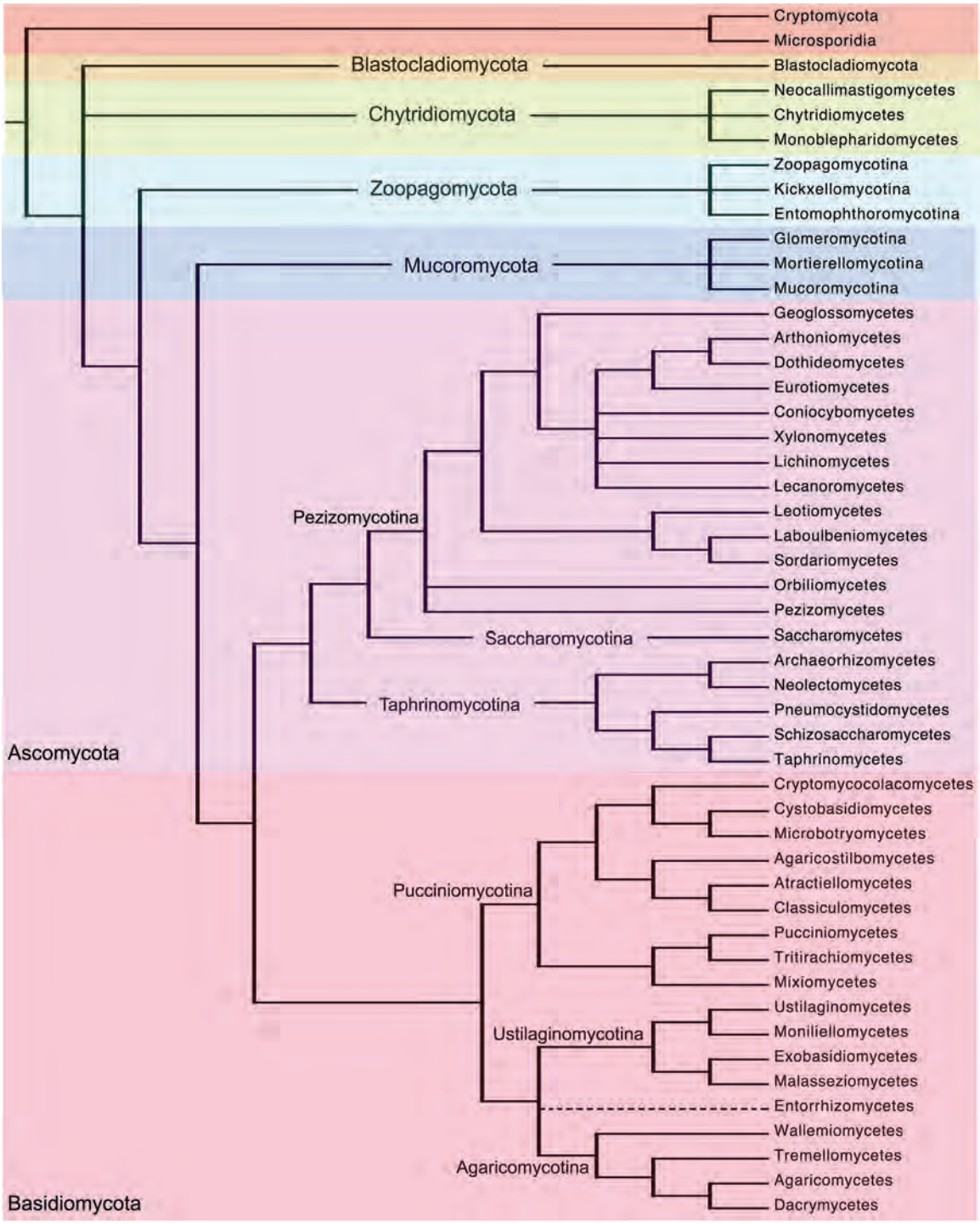


Figure 1 Fungal tree of life. Cladogram of the kingdom Fungi based on published multi-gene and genome-scale phylogenies (11–14, 17, 18, 32, 33, 83, 98, 109, 112, 167, 168). Polytomies represent regions of the tree currently unresolved by molecular and genomic data.

Table 1 Classification of the kingdom Fungi

Cryptomycota M.D.M. Jones & T.A. Richards 2011
[=Rozellomycota Doweld (2011)]
Microsporidia
Blastocladiomycota T.Y. James (2007)
  Blastocladiomycetes Doweld (2001)
Chytridiomycota Hibbett et al. (2007)
  Chytridiomycetes Caval.-Sm. (1998)
  Monoblepharidomycetes J.H Schaffner (1909)
  Neocallimastigomycetes M.J. Powell (2007)
Zoopagomycota Gryganski et al. (2016)
  Zoopagomycotina Benny (2007)
  Kickxellomycotina Benny (2007)
  Entomophthoromycotina Humber (2007)
    Basidiobolomycetes Doweld (2001)
    Neozygitomycetes Humber (2012)
    Entomophthoromycetes Humber (2012)
Mucoromycota Doweld (2001)
  Glomeromycotina Spatafora & Stajich (2016)
    Glomeromycetes Caval.-Sm. (1998)
  Mortierellomycotina Hoffm., K. Voigt & P.M. Kirk (2011)
    Moretierellomycetes Caval.-Sm. (1998)
  Mucoromycotina Benny (2007)
Ascomycota (Berk.) Caval.-Sm. (1998)
  Pezizomycotina O.E. Erikss. & Winka (1997)
    Arthoniomycetes O.E. Erikss. & Winka (1997)
    Coniocybomycetes M. Prieto & Wedin (2013)
    Dothideomycetes O.E. Erikss. & Winka (1997)
    Eurotiomycetes O.E. Erikss. & Winka (1997)
    Geoglossomycetes Zheng Wang, C.L. Schoch & Spatafora (2009)
    Laboulbeniomycetes Engler (1898)
    Lecanoromycetes O.E. Erikss. & Winka (1997)
    Leotiomycetes O.E. Erikss. & Winka (1997)
    Lichinomycetes Reeb, Lutzoni & Cl. Roux (2004)
    Orbiliomycetes O.E. Erikss. & Baral (2003)
    Pezizomycetes O.E. Erikss. & Winka (1997)
    Sordariomycetes O.E. Erikss. & Winka (1997)
    Xylonomycetes Gazis & P. Chaverri (2012)
  Saccharomycotina O.E. Erikss. & Winka (1997)
    Saccharomycetes G. Winter (1880)
  Taphrinomycotina O.E. Erikss. & Winka (1997)
    Archaeorhizomycetes Rosling & T.Y. James (2011)
    Neolectomycetes O.E. Erikss. & Winka (1997)
    Pneumocystidomycetes O.E. Erikss. & Winka (1997)
    Schizosaccharomycetes O.E. Erikss. & Winka (1997)
    Taphrinomycetes O.E. Erikss. & Winka (1997)
Basidiomycota R.T. Moore (1980)
  Agaricomycotina Doweld (2001)
    Agaricomycetes Doweld (2001)
    Dacrymycetes Doweld (2001)
    Tremellomycetes Doweld (2001)
    Wallemiomycetes Zalar, de Hoog & Schroers (2005)
  Pucciniomycotina R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
    Agaricostilbomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
    Atractiellomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
    Classiculomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
    Cryptomycocolacomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
    Cystobasidiomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
    Microbotryomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
    Mixiomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
    Pucciniomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. (2006)
    Tritirachiomycetes Aime & Schell (2011)
  Ustilaginomycotina Doweld (2001)
    Exobasidiomycetes Begerow, M. Stoll & R. Bauer 2007
    Malasseziomycetes Denchev & T. Denchev (2014)
    Moniliellomycetes Q.M. Wang, F.Y. Bai & Boekhout (2014)
    Ustilaginomycetes E. Warming (1884)
  Incertae sedis
    Entorrhizomycetes Begerow, Stoll & R. Bauer (2007)

immersed in their substrate, while only the rhizoids of epibiotic chytrids are immersed. Often, the thalli of single-celled chytrids are converted entirely to thin-walled zoosporangia or thick-walled resting sporangia (holocarpic condition), and others have thalli that are only partially converted to reproductive structures (eucarpic condition). Other terms describe the number of reproductive structures produced by an individual: a single sporangium on a thallus is a monocentric thallus, and multiple sporangia on a rhizomycelium or mycelium are termed polycentric. Zoosporangia, in which zoospores are produced, are asexual reproductive structures; resting sporangia that germinate by release of flagellated cells and resting spores that germinate by germ tubes may be asexual or sexual structures.

Cryptomycota/Microsporidia

Cryptomycota plus Microsporidia are sister to the remaining lineages of Kingdom Fungi. (Note: Rozellomycota [20] is another name for Cryptomycota [21] based on the genus *Rozella* and the principle of autotypification [14]. *Cryptomyces* is a genus in Ascomycota and cannot be used to typify Cryptomycota.) Cryptomycota consists of a handful of described taxa and taxa that are known only from environmental samples. One described taxon is *Rozella* (Fig. 2a), a biotrophic intracellular parasite of other zoosporic fungi

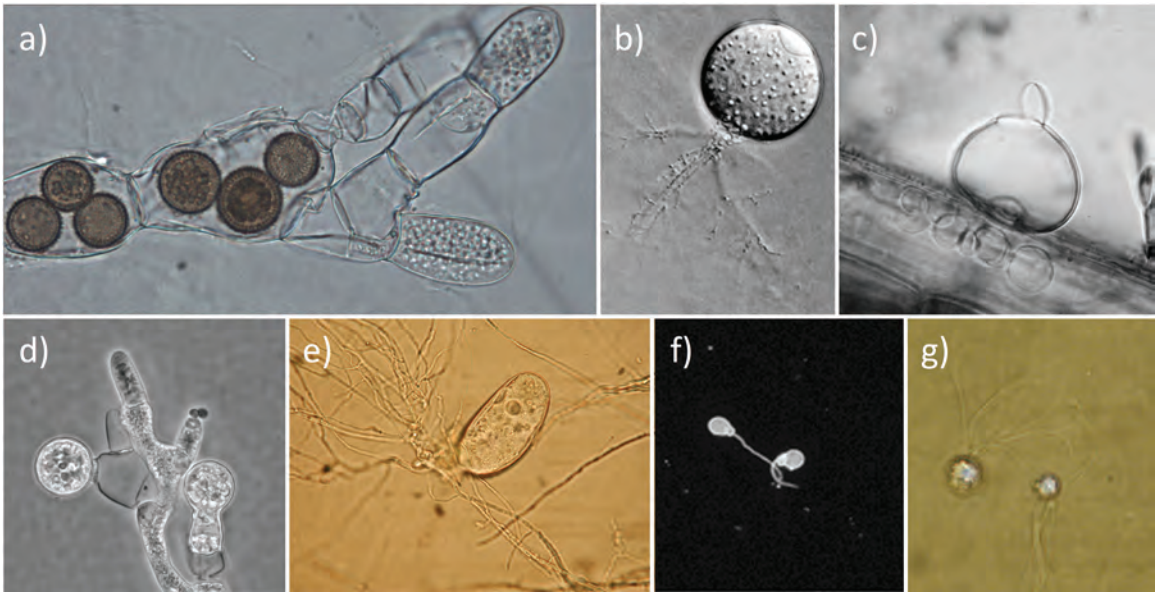


Figure 2 Examples of zoosporic fungal diversity. (**a**) *Rozella allomycis* (Cryptomycota) parasitizing hyphae of *Allomyces* (Blastocladiomycota). Chytridiomycota: (**b**) *C. hyalinus* (Chytridiomycetes) monocentric, operculate zoosporangium with rhizoids; (**c**) *Catenochytridium* sp. (Chytridiomycetes) monocentric, operculate zoosporangium with rhizoids; (**d**) *Monoblepharis polymorpha* (Monoblepharidomycetes) mature zygote or oospore, empty and mature antheridia and antherozoids or male gametes emerging from antheridium (by Marilyn M. N. Mollicone); (**e**) *Neocallimastix* sp. (Neocallimastigomycetes) monocentric thallus with rhizoids (by Gary Easton); (**f**) *Olpidium bornovanus* (*incertae sedis*) zoospores (photo by D'Ann Rochon); (**g**) *Neocallimastix* sp. (Neocallimastigomycetes) multiflagellate zoospores (photo by Gary Easton).

of Chytridiomycota and Blastocladiomycota and oomycetes of the kingdom Stramenopila (22). There are few additional described genera and species of Cryptomycota, but environmental sampling using molecular markers has revealed a phylogenetically diverse assemblage of fungi detected in soils, marine and freshwater sediments, and oxygen-depleted environments (23, 24). Some environmental Cryptomycota produce zoospores with a single, smooth flagellum, but chitin, a cell wall carbohydrate produced by most fungi, was not originally detected in the life history stages first observed (25). A more recent study detected chitin restricted to the cyst phase that attaches to the *Allomyces* hyphae and in the inner wall of the resting sporangia (26). The ecology of the environmental Cryptomycota is largely conjecture at this time, because they have not been cultured, an observation frequently invoked in fungal systems to infer an obligate biotroph. Recently, molecular data have determined that *Paramicrosporidium* (20), nonflagellated parasites of amoebae, and *Amoeboaphelidium*, an algal parasite, are members of Cryptomycota (27), lending support to parasitism as an ecological signature of the phylum.

Microsporidia are intracellular parasites of all major groups of animals. They are particularly well known from insects, crustaceans, and fish but are also known to occur in mammals, including humans (28). Microsporidia produce unique spores that infect host cells through a harpoon-like organelle that pierces the host cell membrane and provides a conduit for the injection of the parasite's cytoplasm (29). Once inside the host cell, a spore, which includes an inner chitin-containing wall, is ultimately formed. The phylogenetic affinity of Microsporidia has been difficult to determine, with past classifications placing it among the polyphyletic protists, and early multigene phylogenies placing it in different parts of the kingdom Fungi (reviewed in reference 30). Genome-scale phylogenies have also proven problematic due to highly reduced genomes and fast rates of nucleotide substitution (31), but multiple analyses have garnered increasing support for its close relationship to Cryptomycota (32, 33). Comparative genomic analyses have demonstrated that both groups share genomic traits including a nucleotide transporter that Microsporidia use for obtaining ATP from their hosts (33). This phylogenetic placement of Microspo-

ridia provides further evidence for the early origins of parasitism and more than one loss of the flagellum within the kingdom Fungi.

Blastocladiomycota

The remaining two phyla of zoosporic fungi are Blastocladiomycota and Chytridiomycota. The branching order of these two lineages is unresolved (Fig. 1), and both have been inferred as the sister lineage to the terrestrial, nonflagellated fungi in large multigene and genome-scale phylogenetic analyses (11, 13, 18). The failure to resolve this branching order appears to be attributed to low phylogenetic signal, rather than strong conflict among competing individual gene trees (18), although only two species of Blastocladiales have been sequenced at this time. Resolution of this branching is central to accurately understanding the changes in life cycles and morphology, especially the loss of flagellum that accompanied the transition onto land. Additional taxon sampling and analysis of changes associated with genome content (e.g., phylogenetic mapping of gene birth events, rare genomic changes, etc.) will be required.

Blastocladiomycota contains a single class and order, Blastocladiomycetes and Blastocladiales, respectively. Fossils of both sporothalli and gametothalli have been described from the Lower Devonian (Rhynie chert) as *Paleoblastocladia* (34), supporting it as an ancient lineage, but current molecular data are consistent with extant species sharing a recent MRCA as compared to other phyla of fungi. Blastocladiomycota exhibit a range of growth morphologies from monocentric with limited thallus development to polycentric with the production of robust, coenocytic hyphae. Furthermore, most known species exhibit a true alternation of generation with free-living haploid and diploid life stages. In *Allomyces*, for example, free-living haploid thalli produce male and female gametangia that can usually be differentiated through color and size; the male gametangia are typically pigmented with carotenoids and are smaller in size than female gametangia. Both male and female gametangia produce motile gametes (planogametes) that fuse to form a diploid zygote, which germinates to form a free-living diploid thallus. This thallus produces sporangia that yield diploid zoospores that are released into the environment and germinate to form other diploid thalli. The diploid thallus ultimately matures and produces resistant sporangia that are the sites of meiosis and production of haploid zoospores, which when released into the environment germinate into haploid thalli, completing the life cycle. Meiosis associated with spore formation (sporic) is unknown in any other fungi.

Well-studied genera within Blastocladiales include *Allomyces*, *Blastocladiella*, *Coelomomyces*, and *Physoderma*, which exhibit saprobic and parasitic ecologies associated with animals and plants. *Allomyces* is frequently isolated from soil and serves as an excellent instructional model of polycentric development (mycelial development with multiple sporangia) and alternation of generations. Its ease of growth on synthetic media with simple sugar sources is consistent with a saprobic life history. *Blastocladiella* is another saprobic species isolated from soil, but it displays monocentric development, with a simple thallus consisting of a single sporangium anchored to its substrate by rhizoidal elements. *Coelomomyces* is a parasite of mosquitoes and copepods, with alternate generations produced in different hosts. Haploid zoospores infect copepods and produce one- to two-celled wall-less hyphal bodies within the host. These hyphal bodies give rise to flagellated gametes that fuse inside or outside of the copepod host, resulting in a diploid flagellated zygote that infects mosquito larvae. Inside the mosquito, the fungus develops a limited diploid thallus that produces resting sporangia, which are the site of meiosis and production of haploid zoospores. *Physoderma* includes stem and foliar pathogens of plants (e.g., corn, alfalfa, etc.), which is relatively rare for zoosporic species of the kingdom Fungi. Species of *Physoderma* produce monocentric epibiotic sporangia that infect a single host cell, but as the infection progresses, polycentric growth develops, producing large endobiotic sporangia that occupy much of the host cell.

Chytridiomycota

Chytridiomycota includes three classes of fungi: Chytridiomycetes, Monoblepharidomycetes, and Neocallimastigomycetes. Although some classifications recognize the latter two classes as phyla (e.g., 14), the three taxa collectively form a well-supported monophyletic clade in genome-scale analyses, but their relationship to each other is uncertain (18). Chytridiomycota may have been the earliest fungi in terrestrial environments, but it is not clear if certain Precambrian microfossils actually represent species of the phylum. Spherules and flask-shaped fossils from chert lenses of the Early Devonian Rhynie chert formation have been interpreted as thalli and zoosporangia of Chytridiomycota. The study of plants and plant parts, including pollen, has been a productive means of discovering and identifying increasing numbers of Chytridiomycota fossils and the presumptive reactions of their hosts 412 million years ago (16).

Chytridiomycetes

Chytridiomycetes are water-inhabiting fungi, often parasitic on algae and oomycetes, or soil inhabitants, some of which are parasitic on vascular plants. A few chytrids parasitize animal eggs and protozoa, while others are saprobic on the decaying remains of plants. Multigene phylogenetic analyses, new culture techniques, and additional collections of Chytridiomycetes have revealed greater diversity and led to increased numbers of orders in which to classify about 700 species in under 90 genera (e.g., 11, 14). Today there are 10 described orders of Chytridiomycetes: Chytridiales, Spizellomycetales, Cladochytriales, Rhizophydiales, Polychytriales, Rhizophlyctidales, Lobulomycetales, Synchytriales, Gromochytriales, and Mesochytriales (e.g., 35–39).

An exemplar life cycle is that of *Chytriomyces hyalinus*, which forms a well-developed rhizoidal system within its substrate (Fig. 2b). The sporangium that develops from the encysted zoospore has a saucer-shaped operculum from which zoospores escape into a fibrous vesicle of overlapping filaments where the cells complete their maturation and then escape (40). The zoospores encyst and germinate to form new sporangia and rhizoids (asexual thalli) or to function as sexual thalli. This is one of the few members of the Chytridiomycetes in which sexual reproduction has been well documented. The rhizoids of the two thalli come into contact and fuse, and a resting spore forms at the junction of the rhizoidal anastomosis. The resting sporangium develops a thick wall and eventually germinates by production of zoospores, apparently after meiosis (41). Other well-studied members of Chytridiomycetes include *Synchytrium endobioticum*, a pathogen that causes potato wart disease, and *Nowakowskiella*, an operculate, polycentric genus of aquatic saprobes of decaying plant materials. Species of *Nowakowskiella* are often isolated from pond water by using cellulosic baits. The most widely studied chytrid is *Batrachochytrium dendrobatidis*, the fungus associated with amphibian declines (42, 43). The disease was first detected in Australia and Panama, but it now has been found on all continents except Antarctica. Estimates predict that 30% of the world's amphibian species will be affected by severe population decline or extinction (44–47). Zoospores attack the keratinized parts of the amphibian, such as larval mouthparts and the first two layers of adult skin, to cause the infection. The flagella are resorbed, and the *B. dendrobatidis* cells enlarge within the infected host cells. Growth of the somatic cells and their conversion to zoosporangia cause the hyperplasia and hyperkeratosis that are symptomatic of the disease. Severe infections reduce the efficiency of cutaneous respiration and osmoregulation. Evolutionary analyses of the *B. dendrobatidis* genome revealed that it has evolved from saprobic ancestors and that its unique ecology of being a vertebrate pathogen is correlated with lineage-specific expansion of multiple gene families of proteases (48).

Monoblepharidomycetes

Monoblepharidomycetes consists of only about 30 species in 6 genera, most of which are saprobes that grow in fresh water on submerged twigs and fruits. The fungi can be isolated by baiting soil samples in water with hemp or sesame seeds. Genera are distinguished in part by a polycentric mycelial thallus (*Gonapodya*, *Monoblepharella*, and *Monoblepharis*) or a uniaxial thallus (*Oedogoniomyces*, *Harpochytrium*, and *Hyaloraphidium*). When hyphae are present in Monoblepharidales, they appear foamy or reticulate because the protoplasm is highly vacuolated. Monoblepharidales is of special interest because of its unique method of sexual reproduction using a nonmotile female gametangium and a flagellated male gamete, unlike any other fungus.

Monoblepharis polymorpha has a well-developed branched thallus consisting of hyphae with a foamy appearance. Elongated zoosporangia are borne singly at the hyphal tips subtended by a septum. Zoospores are released from the tip of the sporangium, swim for a time, become rounded, and germinate by a germ tube to form a new mycelium. The same thallus that bears sporangia also produces gametangia when subjected to higher temperatures. The elongated male gametangium is borne on the large, rounded egg-like female gametangium (Fig. 2d). Uniflagellate gametes formed within the male gametangium mature and are released. A single male gamete fertilizes the enlarged female gamete, resulting in cytoplasmic fusion and production of a diploid zygote. The zygote functions as a resting spore and germinates by producing a hypha to initiate a new thallus. The site of meiosis has not been demonstrated but probably occurs in early divisions of the zygote nucleus (49, 50).

Neocallimastigomycetes

The zoospores of members of Neocallimastigomycetes were once considered to be flagellated protozoa, but DNA sequences placed them in a distinct group of the core chytrids (11). The class consists of about 20 species placed in 6 genera, including *Neocallimastix*, *Orpinomyces*, and *Piromyces* (Fig. 2e). All species inhabit anaerobic regions of the rumen and hindgut of

herbivores, and they produce cellulases and xylanases that help to degrade the dietary fibers from plant cell walls (51, 52). Phylogenomic analyses revealed that many of these plant cell wall-degrading enzymes are of bacterial origin and represent a horizontal gene transfer from bacteria that presumably co-occur with Neocallimastigales in the herbivore digestive tract (53). All species are obligate anaerobes, and although a few other fungi (e.g., *Blastocladia* and certain yeasts in Saccharomycetales) are facultatively anaerobic, among fungi only members of Neocallimastigales require completely anaerobic environments. As a group, these fungi grow on a range of simple and complex carbohydrates and exhibit mixed acid fermentation (54), and renewed attention is aimed at their potential in applied industrial mycology (55).

ZYGOMYCETE FUNGI (Fig. 3)

As mentioned previously, genome-scale phylogenies do not support the monophyly of zygomycetes and reject the zygospore as a synapomorphy for them (13).

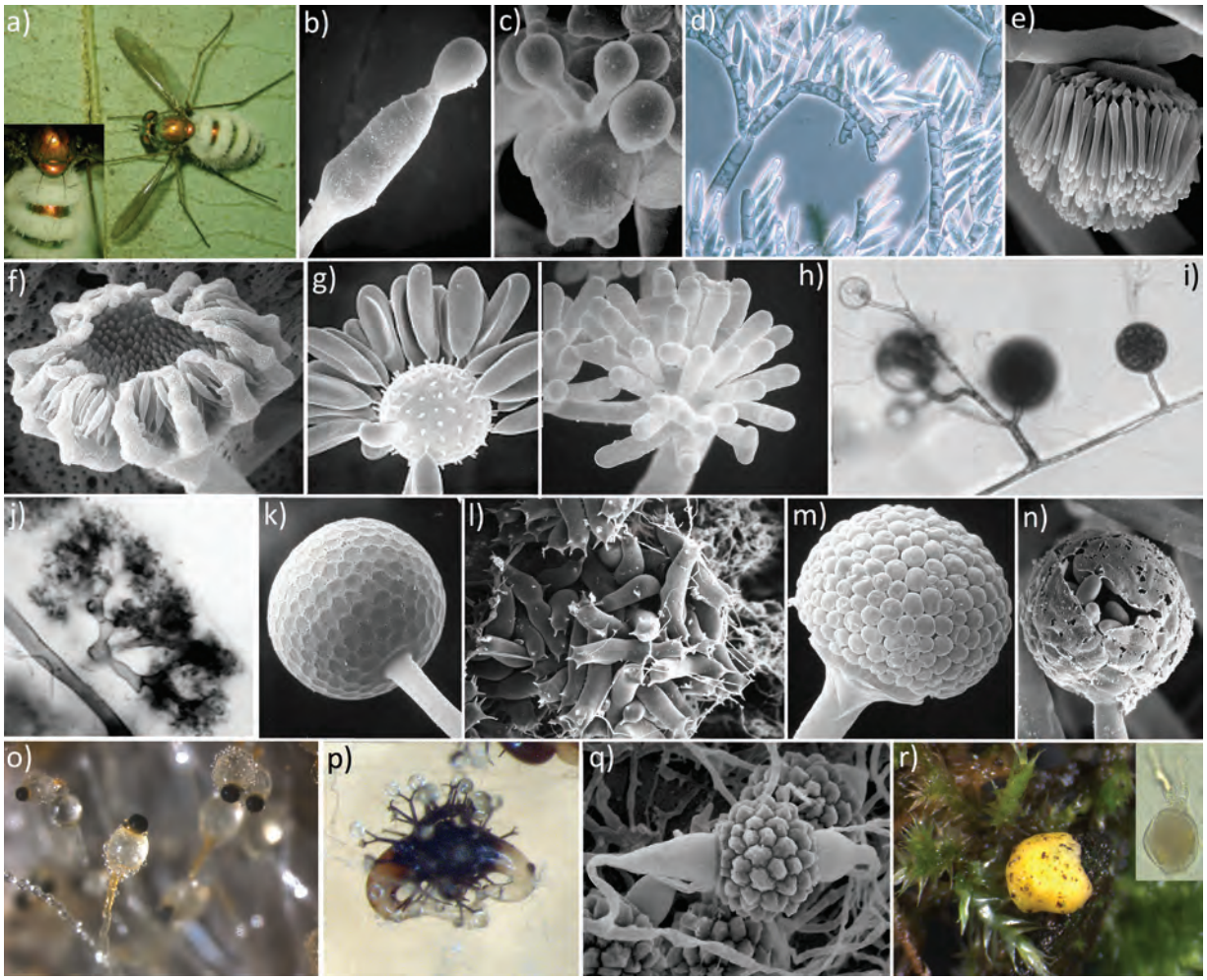


Figure 3 Examples of zygomycete fungal diversity. Zoopagomycota: (**a**) adult fly infected by a species of Entomophthorales; (**b**) *Basidiobolus* conidium; (**c**) *Conidiobolus* conidia; (**d**) *Zygopolaris* thallus with trichospores (photo by R.W. Lichtwardt); (**e**) *Linderina* sporangium; (**f**) *Kickxella* sporangium; (**g**) *Rhopalomyces* sporangium; (**h**) *Piptocephalis* sporangium. Mucoromycota: (**i**) *Glomus* spore (photo from American Society for the Advancement of Science); (**j**) *G. sinuosum* sporocarp (photo by D. Redeker); (**k**) *G. mosseae* arbuscule (photo by K. Wex); (**l**) *Mortierella* sporangium; (**m**) *Lobosporangium* sporangium; (**n**) *Rhizopus* sporangium; (**o**) *Thamnidium* sporangium; (**p**) *Pilobolus* sporangium; (**q**) *Phycomyces* zygosporangium; (**r**) *Cunninghamella* zygosporangium; (**s**) *Endogone* sporocarp, zygospore (inset).

Rather, the zygospore is best interpreted as arising in the MRCA of Zoopagomycota, Mucoromycota, Ascomycota, and Basidiomycota and lost in the MRCA of Dikarya (Ascomycota + Basidiomycota). Most zygomycetes are characterized by coenocytic hyphae and sporangial asexual reproduction, but lineages characterized by septate or compartmentalized hyphae and/or asexual reproduction by formation of conidia exist. Importantly, it is with the emergence of the zygomycete fungi that we observe a loss of the fungal flagellum and the rise of the terrestrial, filamentous fungi. It is generally assumed that this loss of the flagellum in the kingdom Fungi corresponds to the transition to a terrestrial environment and the dawn of early terrestrial ecosystems. As such, zygomycetes represent an important group of fungi for ecological studies of host association and diversification of nutritional modes and cell biology studies regarding the evolution of centrosomes, organelles associated with hyphal growth and differentiation, and multicellularity.

Zoopagomycota

Zoopagomycota is the sister to Mucoromycota+ Dikarya. It comprises three subphyla: Zoopagomycotina, Kickxellomycotina, and Entomophthoromycotina. The primary ecologies of members of the phylum include pathogens and commensals of animals, parasites of other fungi and amoebae, and rarely, as plant associates. The phylogenetic placement of Zoopagomycota as sister to the remainder of nonflagellated fungi is important for numerous reasons, but two are highlighted here. First, diversification with animals and nonplant hosts occurred at least as early as diversification with terrestrial plants. This suggests that fungi were among the first terrestrial organisms and that fossils of the first land animals should be examined with greater scrutiny for fungal associations, potentially providing a more complete picture of early terrestrial fungi. Second, the loss of the flagellum in fungi corresponds to other modifications, including the loss of the centriole. Most nonflagellated fungi of Mucoromycota, Basidiomycota, and Ascomycota possess an organelle unique to fungi, the spindle pole body, which serves as the microtubule attachment necessary for chromosome segregation during nuclear division. It has been hypothesized that the spindle pole body is derived from centrioles through the loss of the 9+2 microtubular system, though there is no support for this homology based on detectable sequence similarity. In contrast, Zoopagomycota lineages retain a functional centrosome that possesses a degenerate 9+2 microtubular system (56). Furthermore, there is some evidence that the genus *Olpidium*, a zoosporic fungus that retains its flagellum and infects nematodes and plant roots of Brassicaceae, may be a member of—or closely related to—Zoopagomycota (12). Collectively, these observations suggest that Zoopagomycota is critical to understanding the evolution of fungi as they transitioned to terrestrial ecosystems.

Zoopagomycotina contains a single order, Zoopagales. Species in this order include predators of nematodes (e.g., *Stylopage*) and nematode eggs (e.g., *Rhopalomyces* [Fig. 3g]), predators of amoebae (e.g., *Stylopage*, *Zoopage*), and mycoparasites of mucoralean fungi (e.g., *Syncephalis*). Hyphae are small in diameter, coenocytic, and they form haustoria on or within their hosts. Asexual reproduction is by conidia or sporangia according to species, and where known, sexual reproduction is by production of zygospores. Many of these fungi are obligate symbionts and thus are difficult to obtain in axenic culture, and for this reason there is a paucity of molecular and genomic data. Furthermore, these fungi are underrepresented in environmental sequence data (57), presumably due to the inadequacy of existing environmental sampling techniques (e.g., primer bias, insufficient reference data, etc.), and their internal transcribed spacer sequences are longer than most fungi, contributing to underrepresentation (58).

Kickxellomycotina comprises four orders: Asellariales, Dimargaritales, Harpellales, and Kickxellales. Species of Kickxellomycotina possess hyphae that are regularly compartmentalized by bifurcate septa that are occluded by a lenticular plug. Asellariales and Harpellales are associated with digestive tracts of aquatic stages of arthropods and comprise two of the four orders that have been treated previously as Trichomycetes (59); the other two orders, Amoebidiales and Eccrinales, are members of Mesomycetozoea, not the kingdom Fungi (60, 61). Asellariales has filamentous, branched thalli and reproduces asexually by disarticulation of the thalli into arthrospores. They occur in the digestive tracts of marine, aquatic, and terrestrial species of isopods and Collembola, where they are thought to function as commensals (62). Harpellales has branched or unbranched filamentous thalli, and they reproduce by trichospores, which are asexual spores with hair-like appendages (Fig. 3d). They attach to the hindgut of aquatic stages of arthropods via a holdfast and are generally considered to be in a commensal relationship with their host (59). Dimargaritales species are haustorial parasites of other fungi, with the best-known species occurring on mucoralean hosts (63), and Kickxellales includes mycoparasites and saprobes isolated from soil (64). Dimargaritales and Kickxellales, as well as Zoopagales, produce unique

sporangia called merosporangia. These are cylindrical sporangia that arise from a bulbous structure, and one or more sporangiospores may occur in chains within the sporangium (Fig. 3e–h).

Entomophthoromycotina contains three classes, each with a single order: Basidiobolomycetes and Basidiobolales, Entomophthoromycetes and Entomophthorales, and Neozygitomycetes and Neozygitales (13, 65, 66). These fungi are associated with animals as either commensals isolated from animal dung or as pathogens and parasites of insects. Many species are commonly isolated from soil and maintained in pure culture, which is consistent with a saprobic life cycle phase. Basidiobolales is commonly isolated from amphibian dung, although species are known to occur on the dung of many vertebrates. They produce primary conidia that forcibly eject a single spore which if it lands on an appropriate substrate will germinate to form a mycelium or otherwise will undergo repetitive germination, producing a secondary conidium (Fig. 3b). Under some conditions nonforcibly discharged capilliconidia are produced from forcibly discharged conidia. These adhere to the outer surface of insects (67). Dispersal is then achieved when the insects are ingested by insectivorous animals, and after surviving gut passage, the fungus is excreted with the feces. In a few cases gut infections are known in various marine and terrestrial vertebrates, and human gut infections have been mistaken for Crohn's disease (68). The ecological function of Basidiobolales is speculative at this time because no definitive functional experiments have been performed, but its specificity to animal dung and placement in Zoopagomycota support an interaction with animal hosts in the digestive system. The phylogenetic placement of Basidiobolales with molecular- and genome-scale data is problematic. In all current data sets, it is characterized by long and unstable branches, and its relationship to other Entomophthoromycotina is unambiguous at this time (69).

Entomophthorales, or literally, insect destroyers, comprises pathogens of insects (Fig. 3a). They infect their hosts via spores and multiply within the host as one- to two-celled hyphal bodies, which also can function as gametangia. Upon the host's death, the fungus ruptures through the cuticle segments, producing forcibly discharged primary conidia (Fig. 3a). Frequently, infected hosts alight in perched or elevated positions, a phenomenon known as summit disease, which is thought to be an induced behavior or adaptation for spore dispersal of the pathogen (20, 70, 71). Neozygitales are pathogens of insects and mites. They were classified as a family within Entomophthorales but were distinguished from Entomophthorales based on the shape and size of chromosomes (65), although inadequate molecular data currently exist to test this hypothesis. *Neozygites* produces adhesive capilliconidia similar to those of *Basidiobolus* (72).

Mucoromycota

Mucoromycota consists of the subphyla Glomeromycotina, Mortierellomycotina, and Mucoromycotina. Unlike Zoopagomycota, Mucoromycota is characterized by plant associations and plant-based ecologies (e.g., mycorrhizae, root endophytes, decomposers, etc.). Some exist as parasites of animals and other fungi, but these all represent opportunistic infections of hosts with compromised immune systems or relatively recent derivations from saprobic ecologies (73). Mucoromycota is the sister group to Dikarya, which is also characterized by dominant plant-associated life styles, suggesting that the MRCA of Mucoromycota and Dikarya corresponds to the origin of modern fungus-plant associations, or at least the evolutionary potential for such relationships.

Glomeromycotina consists of the arbuscular mycorrhizae and *Geosiphon*, a symbiont of cyanobacteria (74). Arbuscular mycorrhizae (Fig. 3i–k) are the most common form of mycorrhizae on the planet, and arbuscule fossils are present among the first land plant fossils (75–77), confirming an ancient symbiosis. As such, they are a central taxon in the development of hypotheses concerning the evolution of early land plants and terrestrial ecosystems (78–80). Despite this importance, they have been an enigma with respect to phylogenetics of the kingdom Fungi. Morphologically, they resemble zygomycetes in the production of coenocytic hyphae and terminal or subterminal spores that resemble azygospores (Fig. 3i), asexually formed zygospore-like structures produced terminally on a single hypha or suspensor cell (81). Sexual reproduction has never been observed for the group, preventing analysis of morphological characters traditionally used in classifications. Early molecular phylogenies based on the small subunit ribosomal DNA resolved the arbuscular mycorrhizae—with varying statistical support depending on the analysis—as separate from the zygomycetes and sister to Dikarya (40, 82). However, genome-scale phylogenies and genome content analyses strongly support the arbuscular mycorrhizae as members of Mucoromycota (13, 83). Recently, mating type genes have been identified in the Glomeromycotina genomes, and their structure and diversity are consistent with a functional sexual reproductive cycle without morphological evidence of sex. Interestingly, the *MAT* genes are more similar to those of basidiomycetes than those in other fungal groups, although this is still

a topic of debate (84). So while we have learned much about Glomeromycotina from genomic data, where and when these fungi undergo sexual reproduction remains a mystery. Currently, there are four orders of Glomeromycotina, Archaeosporales, Diversisporales, Glomerales, and Paraglomerales, with *Geosiphon* being classified in Archaeosporales (74).

The relationship of Glomeromycotina to the other subphyla of Mucoromycota is unresolved, with some analyses resolving it as sister to Mortierellomycotina and Mucoromycotina, while others resolved it as the sister group to Mortierellomycotina. The taxon sampling for both Glomeromycotina and Mortierellomycotina is sparse, and expanded taxon sampling is needed to fully test these rival hypotheses. Mortierellomycotina, and its sole order Mortierellales, are commonly isolated soil fungi. They produce zygospores and sporangia similar to some species of Mucorales (Fig. 3l and 3m), the order in which they were previously classified, but molecular phylogenetics (85) and genome-scale (13) phylogenies both strongly support the taxon as representing a distinct subphylum. Interestingly, even earlier studies of sterols separated the two orders because Mortierellales contain membrane sterols other than ergosterol (86). These fungi have been demonstrated as root endophytes of plants (87, 88), but their effect on the host fitness remains unknown. Some species are reported to have distinctive odors, perhaps associated with animal dispersal. Mortierellales are also prolific producers of fatty acids, in particular arachidonic acid, and *Mortierella* species are used in its industrial production (89). Both Glomeromycotina and Mortierellomycotina possess intimate relationships with bacteria, and while facultative, show high levels of specificity and cospeciation (90, 91), and the fungus tends to grow better when cleared of the bacterium (92). Because of their relationship to Glomeromycotina, demonstrated plant associations, and industrial applications including potential alternative fuels, Mortierellomycotina are the subject of significant genomic inquiries.

Mucoromycotina contains the remainder of known zygomycete species and is classified in three orders: Mucorales, Umbelopsidales, and Endogonales (13). Mucorales is one of the more commonly isolated groups of fungi, because many are fast-growing, early colonizers of carbon-rich substrates. Because many species culture relatively easily, Mucorales are well represented in culture collections, and their zygospores and sporangia (Fig. 3n–r) are well documented (93). They include taxa that cause economically significant pre- and post-harvest diseases of fruits (e.g., *Gilbertella*, *Mucor*, *Rhizopus*). They also significantly impact humans both beneficially through their use in industrial production of food (e.g., tempeh, *Rhizopus*) and compounds used as food supplements (e.g., beta-carotene, *Blakeslea*), and antagonistically as rare but increasingly diagnosed human mycoses (e.g., *Mucor*, *Apophysomyces* [94]). It is among Mucorales that sexual reproduction in fungi was first demonstrated (95), and numerous species of Mucorales exhibit phototropic responses to light (96), making them important eukaryotic model organisms (e.g., *Mucor mucedo*, *Phycomyces blakesleeanus*). Umbelopsidales was recently described for *Umbelopsis* (13), a genus of soil-inhabiting fungi that also occurs as root endophytes (87). Like *Mortierella*, it was formerly classified in Mucorales, but genomic and molecular analyses support it as a distinct taxon (85). Endogonales are saprobic or ectomycorrhizal depending on the species (81). Saprobic species occur in heavily decayed woody substrates, while mycorrhizal species associate with both nonvascular and vascular plants (78). They have been argued as important organisms in the colonization of land by green plants (79) and represent an independent origin of mycorrhizae relative to both Glomeromycotina and Dikarya. It is within Mucoromycota that the first multicellular sporocarps were produced. These include independent origins in *Endogone* (Mucoromycotina [78]) (Fig. 3s) and *Modicella* (Mortierellomycotina [97]), and as aggregations of spore-producing hyphae and spores in species of Glomeromycotina (Fig. 3j) (74). Multicellular sporocarps are not produced by Zoopagomycota, suggesting that the genetic potential for complex thallus development did not arise until the MRCA of Mucoromycota and Dikarya and then resulted in multiple independent inventions of complex spore-producing structures in Mucoromycota, Ascomycota, and Basidiomycota (98).

DIKARYA

Dikarya is the only described subkingdom of Fungi and comprises the phyla Ascomycota and Basidiomycota. The name is based on the nuclear condition of possessing two genotypically distinct nuclei within the thallus at some point in the life cycle. The hyphae of Dikarya are regularly septate, and if a hyphal compartment contains one nucleus or more than one nucleus but all of the same genotype, it is referred to as monokaryotic or homokaryotic, respectively. If a hyphal compartment contains two nuclei of different genotypes, it is referred to as dikaryotic or heterokaryotic. In Basidiomycota, basidiospores typically possess one type of nucleus and germinate to form a mono-

karyon. Monokaryons fuse (plasmogamy) with other compatible monokaryons, producing the dikaryotic state that is maintained by the products of the mating type genes. The dikaryon constitutes the major vegetative phase of the life cycle for most Basidiomycota, and it is as a dikaryon that most Basidiomycota function in nature (e.g., wood decay, mycorrhizae, pathogens, etc.). Karyogamy occurs in unique cells, termed basidia, resulting in a short-lived diploid zygote that immediately undergoes meiosis to produce haploid nuclei that are incorporated into basidiospores. Importantly, plasmogamy is separated from karyogamy and meiosis both temporally and spatially. In Ascomycota the dikaryotic state is restricted to the sexual reproductive cells, with the vegetative mycelium being homokaryotic. Female gametangia (ascogonia) are fertilized by male gametangia (antheridia) or gametes (spermatia), resulting in a dikaryotic state (ascogenous hyphae). A number of rounds of conjugate nuclear divisions occur prior to karyogamy. Karyogamy and meiosis follow shortly afterward in the young ascus cell, resulting in homokaryotic ascospores. Thus, although both phyla of Dikarya have inherited the dikaryotic state from a common ancestor, they are expressed differently in the life cycles of Ascomycota and Basidiomycota. For more on life cycles of Ascomycota and Basidiomycota, the reader is directed to general mycology textbooks (e.g., 41).

More genomes have been sequenced for species of Ascomycota and Basidiomycota than other phyla of the kingdom Fungi (http://genome.jgi.doe.gov/fungi/), resulting in an increased resolution of phylogenetic relationships and evolutionary processes that have shaped phylogenetic and ecological diversity in Dikarya. Ascomycota consists of three subphyla—Taphrinomycotina, Saccharomycotina, and Pezizomycotina—as does Basidiomycota: Pucciniomycotina, Ustilaginomycotina, and Agaricomycotina. These subphyla include the majority of described species of fungi, and most are associated with plants as symbionts or decomposers of plant materials, although there are numerous independent transitions to other hosts and materials (e.g., animals). It is within Dikarya that we see the apex of multicellular sporocarp production, complex multicellularity, yet sporocarps are distributed sporadically across separate subphyla. Major innovations in sporocarp formation can be found primarily in Pezizomycotina and Agaricomycotina (98), but also within a single genus of Taphrinomycotina (*Neolecta* [99]) and scattered within Pucciniomycotina (e.g., *Septobasidium* [100], *Eocronartium*, and *Jola* [101]). When considering the diversity across the kingdom Fungi, there are a minimum of seven clades of sporocarp-forming fungi: three in Mucoromycota (discussed above), two in Ascomycota, and two in Basidiomycota. Genomic analyses of evolutionary development, or EvoDevo, of sporocarp formation are in their infancy, but a recent study involving Ascomycota paints a complex picture (102). Genes hypothesized to function in complex multicellularity were present in the MRCA of Ascomycota and diversified in Pezizomycotina but were lost in parallel in Saccharomycotina, the budding yeasts, and the yeast lineages of Taphrinomycotina. While present, genes that may be necessary for complex multicellularity did not expand in copy number or show substantial diversification within *Neolecta*, thus complicating a simple explanation of gains and losses of sporocarps.

Ascomycota (Fig. 4)

Ascomycota is a diverse phylum of fungi that includes decomposers associated with a myriad of substrates (e.g., dung, wood, soil), symbionts and associates of plants and animals, and inhabitants of marine and terrestrial ecosystems. Plant-associated species range from antagonistic pathogens to beneficial symbionts (e.g., mycorrhizae) to foliar, root, and wood endophytes whose true functions remain unknown. They have impacted our civilization since the dawn of humans with both positive and negative outcomes. Some ascomycetes were among the first organisms domesticated by humans and have been used in fermentation of foods and beverages for over 9,000 years (103). They are the source of numerous lifesaving drugs including antibiotics, statins, and immunosuppressants. Unfortunately, they are also the causal agents of disease, especially of plants, that have changed continents by removing entire species from the landscape after introduction by humans (e.g., chestnut blight [104]) or have resulted in billions of dollars of loss in modern agriculture (e.g., *Fusarium* head blight [105]). Indeed, our relationship with Ascomycota is one that has profound effects on our planet and our species.

Taphrinomycotina

The subphylum Taphrinomycotina includes about 140 species with both yeast and filamentous growth forms and is a sister group to the remaining Ascomycota. Taphrinomycotina is classified into five classes —Taphrinomycetes, Schizosaccharomycetes, Pneumocystidiomycetes, Neolectomycetes, and Archaeorhizomycetes—each with a single order. Taphrinomycetes includes plant pathogens such as *Taphrina* and *Protomyces*, which are characterized by saprobic, monokaryotic yeast and pathogenic, dikaryotic filamentous life stages. Taphrinales has played an important role in



Figure 4 Examples of Ascomycota diversity. (**A**) Apothecia (yellow) of *Orbilia*, Orbiliomycetes (J. H. Petersen/MycoKey). (**B**) Apothecia of *Aleuria*, Pezizomycetes (J. H. Petertsen/MycoKey). (**C**) Thallus of *Ophioparma* with apothecia, Lecanoromycetes (B. McCune, Oregon State University). (**D**) Thallus of *Lichinella*, Lichinomycetes (B. McCune, Oregon State University). (**E**) Bitunicate asci of *Thaxteriella*, Dothideomycetes (S. Huhndorf, Field Museum). (**F**) Thallus of *Arthonia* with apothecia, Arthoniomycetes (B. McCune, Oregon State University). (**G**) Thallus of *Prolixandromyces*, Laboulbeniomycetes (A. Weir, SUNY-ESF). (**H**) Perithecia of *Neurospora*, Sordariomycetes (N. B. Raju, Stanford University). (**I**) Earth-tongue apothecia of *Cudonia*, Leotiomycetes (Z. Wang, Iowa State University). (**J**) Cleistothecia of *Eupenicillium*, Eurotiomycetes (D. Geiser, Penn State University). (**K**) Operculate ascus of *Peziza* (J. H. Petersen/MycoKey). (**L**) Ascostroma of *Venturia*, Dothideomycetes (T. Volk, University of Wisconsin at La Crosse). (**M**) Unitunicate asci *Neurospora* (N. B. Raju, Stanford University). (**N**) Prototunicate ascus of *Eurotium* (D. Geiser, Penn State University).

the development of evolutionary hypotheses of the kingdom Fungi and has been considered a possible evolutionary link between Ascomycota and Basidiomycota, due to the life cycle described above, which is similar to that of some smut and other microfungal basidiomycetes of Ustilaginomycotina and Pucciniomycotina. Based on numerous molecular phylogenetic studies, however, it is strongly supported as a member of Ascomycota, and it is unclear if the similarity in life cycles with some Basidiomycota is homologous or not.

Schizosaccharomycetes includes the fission yeasts, which display an unusual form of cell division for fungi. Rather than dividing by budding, as is true for all other yeast forms, cells of Schizosaccharomycetales elongate and divide into equal cells through the formation of a fission plate. *Schizosaccharomyces pombe* is the best-studied species and was originally isolated from millet beer in East Africa (106). Pneumocystidiomycetes comprises pulmonary pathogens of mammals, including humans. Species of *Pneumocystis* are found in soils associated with rodent dens. *Pneumocystis jirovecii* is an opportunistic pathogen of humans and is the causal agent of pneumocystic pneumonia in people with compromised immune systems. Neolectomycetes includes the only sporocarp-producing species of Taphrinomycotina. The genus *Neolecta* forms earth tongue-like sporocarps with a hymenium of asci produced from uniquely branched hyphae (107). The ecology of the group is unknown, but the inability to maintain it in axenic culture suggests a symbiotic association in nature (108). Ancestral character state reconstruction analyses resolved *Neolecta* as an independent origin of sporocarp formation in Ascomycota, unique to that of Pezizomycotina (98, 109), but comparative genomic analyses support the possession of common genetic multicellular machinery between *Neolecta* and Pezizomycotina (102). Archaeorhizomycetes is the most recently described class of Taphrinomycotina. It is commonly detected in environmental sampling of soil and was initially known informally as Soil Clone Group 1 (110, 111). One species was serendipitously described from a culture collection of root-associated fungi; it and a second recently described species are associated with the surfaces of tree roots (112, 113).

Saccharomycotina

The subphylum Saccharomycotina includes about 1,000 species in a single order, Saccharomycetales, and 10 major families and several undescribed clades (114). Saccharomycetales include the majority of the ascomycete yeasts and are characterized by budding in asexual reproduction (115). Yeasts, however, have evolved multiple times in the kingdom Fungi, most likely through parallel diversification of novel transcription factors (116). In Saccharomycotina, somatic cells and hyphae may be haploid or diploid, and diploid cells undergo meiosis to convert to gametes, which fuse to form a zygote and develop into asci to produce one to eight ascospores. Ascogenous hyphae are not produced as in Pezizomycotina. Two other traits that distinguish Saccharomycetales yeasts from other ascomycetes occur during ascosporogenesis. These are meiotic divisions with perpendicular spindles developing within the nuclear envelope and formation of ascospores within a common ascus vesicle instead of ascospores developing in individual ascus vesicles as in Pezizomycotina (41). Although they are relatively similar in morphology, Saccharomycotina yeasts are metabolically diverse. In common with Taphrinomycotina, Saccharomycotina lack the septal pore-blocking Woronin bodies characteristic of Pezizomycotina. Earlier yeast taxa were distinguished by the application of about 100 physiological tests. Previously, hypotheses of relationships were difficult to address because of the lack of distinctive morphological characteristics. Now DNA analysis distinguishes yeast species rapidly, as well as providing phylogenetic hypotheses (114, 115). To expand our knowledge of biotechnologically important yeasts, a variety of additional species were sequenced across the known phylogeny, especially from outside of the better-known *S. cerevisiae* clade. This study revealed several transitions in the early evolution of the ascomycetes, the synteny of the *MAT* loci across Ascomycota, and a genetic code change from CUG-Ser to CUG-Ala in one lineage (117, 118). Phylogenomic analyses have also revealed that *S. cerevisiae* and its relatives are the product of a whole-genome duplication (119, 120) that is the result of an ancient hybridization event between two ancestral species (121).

Yeasts occupy a wide variety of natural (e.g., desert and forest plants, insect guts, and environments) and human-provided (e.g., pickle vats, breweries) habitats. *S. cerevisiae* is the most widely known yeast in the world due to its role in bread making and brewing of alcoholic beverages and as a model organism. This was the first eukaryotic species to have its entire genome sequenced, which was justified by its industrial and biological importance. *S. cerevisiae* and many other yeasts are closely associated with insects, including bees, dipterans, and beetles. Certain yeasts display specific associations with insects and plants, including dipterans and cacti in American deserts (122), nitidulid beetles and ephemeral flowers around the world (123), and widely distributed wood-feeding beetles (124). A dis-

tinctive group of previously unknown yeasts related to *Suhomyces tanzawaensis* are associated with fungus-feeding beetles and drosophilids (125). Sexual reproduction and overwintering of *S. cerevisiae* and its hybrids are promoted in the gut of social wasps (126). The bodies of human beings and many mammals are substrates for animal pathogenic yeasts. *Candida albicans* and relatives inhabit the digestive tracts of healthy individuals but may become invasive in those with suppressed immune systems. Infections can be localized (esophageal candidiasis, or thrush; genital candidiasis, or "yeast infection") or invasive in the brain, heart, bones, and eyes. *Candida* can be spread in the blood, a fairly common infection in hospitals where candidemia has been estimated to be fatal in as many as 19 to 24% of cases (127). *Candida auris*, recently recognized as an emerging fungal disease in the United States, is multidrug-resistant (https://www.cdc.gov/fungal/diseases/candidiasis/candida-auris.html). Few yeasts are plant pathogens, but *Ashbya gossypii* is well known to be destructive to fruit development in cotton (128). This species is also industrially important because it overproduces riboflavin (129). Interestingly, *A. gossypii*, and occasionally *S. cerevisiae*, are insect-associated.

Pezizomycotina

The subphylum Pezizomycotina contains many more species than the two other ascomycete subphyla combined; the 63,000 or so species are placed in 13 classes and 67 orders. Pezizomycotina are mostly filamentous (130), although many are capable of dimorphic growth. With the exception of *Neolecta* (Taphrinomycotina), they include all of the sporocarp-forming Ascomycota, but the life cycles of many are only known from their hyphal and asexual reproductive states. Past classifications of the group have emphasized sporocarp morphology and development and ascus morphology and dehiscence. Ascomycete sporocarps, or ascomata, were categorized into four basic types. Apothecia have an exposed hymenium of asci and are typically cup-shaped or spathulate (Fig. 4a–d). Perithecia enclose the hymenium in a flask-shaped ascoma, and the ascospores are released through an opening called an ostiole (Fig. 4h). Cleistothecia completely enclose an ill-defined hymenium of scattered asci, and no pore or opening is present (Fig. 4j). (Note: Chasmothecia are another form of completely enclosed ascomata, but the hymenium exists as a single basal fascicle of asci.) All three forms of these ascomata are assumed to be formed after fertilization of the ascogonium, a form of development called ascohymenial. The fourth major group of ascomata is ascostromata (Fig. 4l). In these fungi, ascogonia are fertilized in preformed locules, which mature into ascostromata containing asci and ascospores, a form of development called ascolocular.

Asci of Pezizomycotina may be operculate or inoperculate. The apex of operculate asci contains a preformed lid, like a manhole cover, that is opened for ascospore release (Fig. 4k), while inoperculate asci lack an operculum. Inoperculate asci can be further categorized based on the nature of the ascus walls. Bitunicate or fissitunicate asci possess more than one functional wall layer, an ectoascus and an endoascus (Fig. 4e). Ascospores are released by the endoascus rupturing through and extending beyond the ectoascus in a manner reminiscent of a jack-in-the-box. Unitunicate asci possess a single functional wall layer, and the ascus apex usually possesses a pore or canal through which ascospores are released (Fig. 4m). Prototunicate asci are typically globose and possess a thin ascus wall that breaks down, or deliquesces, at ascospore maturity (Fig. 4n). Species that produce prototunicate asci do not forcibly eject their ascospores. The combinations of ascomatal and ascal characters can be informative in describing the overall morphology of a species, but most traits have been gained and lost multiple times during the evolution of Pezizomycotina, with a few notable exceptions (e.g., the operculate ascus of Pezizomycetes).

In addition to the sexual morphs, Pezizomycotina reproduce prolifically through asexual reproduction in which the nuclei of spores are products of mitotic divisions. A species can only have one sexual state but may have many asexual states, a phenomenon called pleomorphy. Many Pezizomycotina are only known from their asexual morphs, and it is common for a species to reproduce asexually throughout the growing season but reproduce sexually only once. Historically, unique Latin binomials were given to asexual and sexual states, a system referred to as the dual system of nomenclature, and thus a single species could have several names. The reason for this was manifold, but the main argument was that the sexual and asexual states of Pezizomycotina could be separated in space and time, so when one observed an asexual state, it could be difficult to link it to a sexual state. Perhaps the fungus was only observed in its asexual form by chance; perhaps the sexual form occurs on a different host or in a different geographic region; perhaps the fungus only reproduces asexually in axenic culture; or perhaps the fungus is truly asexual. The advent of molecular data and phylogenetic analyses provided a mechanism to link sexual and asexual morphs and to identify them as conspecific. For this reason, the dual system of nomen-

clature was abolished recently (131), and all fungi must now be called by only one name. Needless to say, determining what name to call a fungus that has many names is no trivial task and is the subject of much debate, but today students only need to learn one name for a single species.

Nonlichenized apothecial fungi are classified into four classes of Pezizomycotina: Orbiliomycetes, Pezizomycetes, Leotiomycetes, and Geoglossomycetes. Orbiliomycetes and Pezizomycetes are sister to the remainder of the subphylum, but their branching order is unresolved (132). Orbiliomycetes produce small apothecia with inoperculate asci. Apothecia typically fruit on wood or soil, and the asci are small and release ascospores through an apical slit. The group is best known for its asexual states of *Arthrobotrys*, which are predators of nematodes. These fungi form hyphal rings or loops that constrict and capture nematodes as they crawl through the rings. Pezizomycetes produce a diversity of ascomatal types with operculate asci. Apothecia may be minute, as in the coprophilous *Ascobolus*, or large, as in the type genus *Peziza*. Some taxa produce apothecia elevated on a stipe, as in *Helvella*, or below ground, as in the prized culinary truffles of *Tuber*. The asci of all these taxa are operculate and have a positive amyloid reaction, with the exception of species that form truffles, which have lost their ability to forcibly discharge their spores. Leotiomycetes is the largest class of inoperculate apothecial fungi (133). They produce a myriad of apothecial morphologies ranging from cup fungi to stipitate earth tongues to hysteriate apothecia that are resupinate and expose the hymenium as a long and slender slit. At one time all fungi that produce apothecia with inoperculate asci were classified in Leotiomycetes. Species of the class include important plant pathogens (*Sclerotinia*), foliar (*Rhytisma*) and root (*Phialocephala*) endophytes, and mycorrhizae of ericaceous plants (*Rhizoscyphus*), to name a few. Animal pathogens are also known, including the recently emerged white nose syndrome of bats (*Pseudogymnoascus destructans*), which is devastating brown bat populations of North America (134). Geoglossomycetes comprise a subset of earth tongue species (Fig. 4i). These fungi were classified in Leotiomycetes along with other earth tongue genera *Leotia* and *Spathularia*. Molecular systematic analyses revealed that *Geoglossum* and its relatives represent a separate origin of the earth tongue morphology, however, and a unique class of fungi (135).

Sordariomycetes includes nonlichenized, perithecial species with inoperculate, unitunicate asci, but species possessing cleistothecia and/or prototunicate asci are known to be derived from within perithecial lineages (133). Perithecia may be produced on well-developed, stipitate stromata (e.g., as in *Xylaria* and *Cordyceps*), embedded in resupinate (e.g., *Hypoxylon*) or cushion-shaped stromata (e.g., *Hypocrea*), or superficially in an aggregated or scattered manner (e.g., *Neurospora*, Fig. 4h). Species of the class include some of the most devastating plant pathogens known. Chestnut blight, *Cryphonectria parasitica*, essentially eliminated the American chestnut from the forests of eastern North America and contributed to the development of the Plant Quarantine Act of 1912. Dutch elm disease, *Ophiostoma ulmi*, devastated the American elm and significantly impacted its presence and abundance on the landscape and its use as an important urban tree (136). *Fusarium graminearum*, head scab of wheat, is a global pathogen of wheat, one of the most important grains in human agriculture, and results in losses of billions of dollars in crop production (105). In addition to plant pathogens, Sordariomycetes are frequently identified as endophytes through culture and amplicon-based environmental sampling studies (137). Sordariomycetes also includes insect pathogens of *Beauveria* and *Metarhizium*, which are some of the most promising biological control agents of insect pests in agricultural ecosystems (138). Some species have recently been identified as having the potential to grow as an endophyte, suggesting a role in plant protection against insect pests and a nutritional role of transferring nitrogen to the plant host (e.g., 72, 139).

Eurotiomycetes are cleistothecial species that produce prototunicate asci and ascostromatic species that produce bitunicate or fissitunicate asci (140). Cleistothecial taxa include important industrial and medical species of *Aspergillus* and *Penicillium* (Eurotiales) and human pathogens of *Coccidioides* (Onygenales) that cause valley fever. These fungi produce both some of the most potent mycotoxins known to science (aflatoxins of *Aspergillus*) and lifesaving antibiotics that changed the course of history (penicillin of *Penicillium*). There even exists an independent origin of ectomycorrhizae among Eurotiomycetes, the genera *Elaphomyces* and *Pseudotulostoma*, which form large truffle-like or stipitate-capitate fruiting bodies, respectively (141). Although *Pseudoulostoma* and *Elaphomyces* are members of Eurotiales, the carbohydrate metabolism of *Elaphomyces granulatus* has been shown to be more similar to animal pathogens in Onygenales than to other members of its order (142). Ascostromatic species include the black yeasts (Chaetothyriales) that result in opportunistic cutaneous and subcutaneous infections. Lichenized species are also represented among the Eurotiomycetes by the order Verrucariales.

These lichens produce perithecioid ascostromata with bitunicate to fissitunicate asci, representing one of the rare combinations of reported ascohymenial development and bitunicate asci. A third and unique clade of Eurotiomycetes is Mycocaliciales, which are saprobes or commensals on lichens (143).

Dothideomycetes is a large and ecologically diverse class and represents the core of ascostromatic fungi with bitunicate asci (144). Ascostromata are frequently perithecioid in appearance and are referred to as pseudothecia (Fig. 4l), but there are apothecioid and cleistothecioid morphologies, as well. The majority of species are associated with terrestrial plants, and they are commonly identified as endophytes in environmental sampling studies based on cultures and DNA barcodes (e.g., 137, 145, 146). Numerous taxa diverged from this ecology, however, including marine fungi of mangroves, insect pathogens (e.g., *Myriangium*), mycoparasites (e.g., *Ampelomyces*), lichens (e.g., *Trypethelium*), and ectomycorrhizae (147). *Cenococcum geophilum* is one of the most abundant ectomycorrhizae-formers on the planet and represents another independent origin of ectomycorrhizae within the kingdom Fungi (148). Functional genomic studies have demonstrated that *C. geophilum* plays a role in drought tolerance of host trees, where it manipulates the host's response to water stress (149). The class is best known, however, for its large number of virulent plant pathogens including *Bipolaris maydis* (the causal agent of southern corn blight), *Zymoseptoria tritici* (Septoria wheat blotch), and *Mycosphaerella fijiensis* (black sigatoka of banana), to name a few. Species and races of these fungi frequently produce host-selective toxins that function as pathogenicity factors for selective host species and genotypes (150, 151).

Because of their importance in agriculture and relative ease of culturing, more than 100 Dothideomycetes genomes have been sequenced (http://genome.jgi.doe.gov/programs/fungi/). Published analyses of Dothideomycetes genomes revealed that the structural evolution of chromosomes is mostly a product of intrachromosomal rearrangements, a phenomenon called mesosynteny (152). Furthermore, disposable chromosomes, which may be present or absent within an isolate, are common, but their function is mostly unknown. Comparative analyses of multiple genomes across a phylogenetic and ecological diversity of Dothideomycetes support the idea that the plant pathogen "play book" is particularly rich in enzymes for secondary metabolite production, carbohydrate-active enzymes, small secreted proteins (SSPs), peptidases, and lipases (153). In addition, genes that encode for effector proteins, which have a role in pathogenicity, occur often in close proximity to transposable elements (154), suggesting that transposable elements may play a role in their evolutionary diversification.

The remaining two classes of nonlichenized Pezizomycotina are Laboulbeniomycetes and Xylonomycetes. Laboulbeniomycetes is an enigmatic class that is primarily composed of ectoparasites of insects (155–157). In Laboulbeniales, minute thalli develop from an ascospore through a definitive number of cell divisions and adhere to the exoskeleton through the production of a holdfast cell (Fig. 4g). Certain species tend to be host- and position-specific, with some species being transmitted through behaviors associated with sexual reproduction (158). Members of the second order, Pyxidiophorales, are mostly mycelial mycoparasites with ascospore dispersal by arthropods (159). Xylonomycetes is one of the more recently described classes of Pezizomycetes and contains wood endophytes in *Xylona* (160) and endosymbionts of beetles in *Symbiotaphrina*, the latter of which may benefit the insect by detoxifying noxious plant compounds (161).

The remaining four classes of Pezizomycotina are Arthoniomycetes, Coniocybomycetes, Lecanoromycetes, and Lichinomycetes and consist almost entirely of lichenized species (144, 162). Lichens are stable symbioses between a fungus (mycobiont) and photosynthetic green alga and/or cyanobacterium (photobiont) that result in the formation of a thallus unique to the symbiosis. Lichens are some of the more successful fungal symbioses on the planet and are conspicuous members of terrestrial ecosystems, where they play important roles in carbon and nitrogen cycles. The classic definition of a lichen being formed by one fungus has been challenged recently, however, by environmental sampling and microscopy data (163) which demonstrated that basidiomycete yeasts of Cystobasidiomycetes (Pucciniomycotina) were embedded in the cortex. Furthermore, the presence and abundance of the yeasts correlated with variations in phenotype. Although there are fewer genomic studies of lichenized than nonlichenized fungi, current comparative genomic analyses have shed some light on the formation and maintenance of lichen symbiosis. For example, species of lichenized Pezizomycotina possess an ammonium transporter/ammonia permease family that was horizontally transferred from Archaea but is absent in nonlichenized species (164). Functional analyses using the lichen-forming fungus *Endocarpon pusillum* and its algal partner identified genes involved in the nitrogen and carbon transfer between symbionts and lectins required for symbiotic recognition (165).

Lecanoromycetes is the largest and best-studied class of lichens (162). It includes most of the common forest lichens and such growth forms as foliose, fruticose, and crustose lichens. Lichinomycetes and Coniocybomycetes are two smaller classes that form a monophyletic group. Lichinomycetes form gelatinous thalli in which the cyanobacterial photobiont is not sequestered in a defined layer of the lichen. Coniocybomycetes form mazedia, which are stalked ascospore-producing structures. Mazedial lichens are also known from Caliciales of Lecanoromycetes, and mazedia are known from several groups of nonlichenized Pezizomycotina (166). Arthoniomycetes is the second-largest class of lichens, and they possess bitunicate asci, although their ascomata have been interpreted as ascohymenial (144).

Molecular systematics has provided great clarity to the phylogenetic relationships of the classes of lichenized and nonlichenized Pezizomycotina (109, 167), but genomic sampling of lichens is still sparse and is heavily biased toward nonlichenized fungi. Orbiliomycetes and Pezizomycetes are sister to the other classes of Pezizomycotina, with the remaining classes informally referred to as Leotiomyceta to designate their monophyly. Within Leotiomyceta, Sordariomycetes, Laboulbeniomycetes, and Leotiomycetes form a clade, as do Arthoniomycetes and Dothideomycetes. Eurotiomycetes is sister to Arthoniomycetes+Dothideomycetes, and the three classes may form a more inclusive clade with Lecanoromycetes, Coniocybomycetes, Lichinomycetes, and Xylonomycetes, but the relationships are not unequivocally supported by current data (109, 167). Finally, Geoglossomycetes appears to represent an isolated lineage of Pezizomycotina (135), but genomic data are lacking.

Basidiomycota (Fig. 5)

The phylum Basidiomycota is defined by the synapomorphies of basidium and basidiospore. Basidia are modified terminal hyphal cells that are the site of karyogamy and meiosis. They are typically produced in hymenial tissues such as gills or pores. Basidiospores are, with few exceptions, formed on sterigmata, outgrowths of basidia, and typically contain a single haploid nucleus. Basidiospores can either be forcibly ejected from the sterigma (ballistospores) or passively dispersed (statismospores) by water, wind, or animals. Most Basidiomycota have a filamentous thallus that is compartmentalized by regularly distributed septations. Basidiomycota consists of three subphyla: Pucciniomycotina, Ustilaginomycotina, and Agaricomycotina. Although reconstruction of the earliest nodes of Basidiomycota has been problematic, genomic studies tend to support Ustilaginomycotina (smuts and relatives) and Agaricomycotina (fleshy basidiomycetes) as a monophyletic group that shares an MRCA relative of Pucciniomycotina (e.g., 168). Pucciniomycotina includes yeasts and filamentous taxa, with the best-known species being the plant-pathogenic rusts of Pucciniales. Pucciniomycotina and Ustilaginomycotina were formerly classified in the obsolete class Teliomycetes based on the production of teliospores, which germinate to produce basidia (as promycelia) and basidiospores, rather than the production of a hymenium.

Former higher-level classifications of Basidiomycota emphasized the morphology of the basidia and the nature of basidiospore germination. Phragmobasidia were described as basidia with some form of septation, whereas holobasidia lacked septations (169). Heterobasidiomycetes produced basidiospores that could germinate either directly via a germ tube or indirectly via secondary spore production or repetitive germination. Homobasidiomycetes produced basidiospores that germinated directly through germ tube formation only (170). There is considerable positive correlation between the two systems in that most taxa that produce phragmobasidia are heterobasidiomycetes, and most taxa that produce holobasidia are homobasidiomycetes, but with exceptions. When considered in light of current phylogenetic classifications, heterobasidiomycetes are found in all three subphyla of Basidiomycota, and homobasidiomycetes are predominantly found in Agaricomycotina. Interestingly, the current phylogenetic classification of subphyla is most consistent with septum morphology (171, 172). Pucciniomycotina possess a simple septum. Agaricomycotina possess a dolipore septum characterized by a septum wall that swells near the septal pore and is bounded by a septal pore cap that is derived from the endoplasmic reticulum. Ustilaginomycotina possess a range of septum types from simple pores coupled with membranous pore caps to swollen pore margins that lack a septal pore cap.

One final important trait in Basidiomycota taxonomy that we will emphasize here is the clamp connection. Most basidiomycetes possess a vegetative mycelium that is dikaryotic, meaning that each hyphal compartment contains two nuclei. Clamp connections occur in the dikaryon and function in maintenance of the dikaryotic nuclear state. Clamp connections are short, modified hyphal branches that grow away from the hyphal tip and undergo self-fusion with the main hyphae from which they branched. Clamp connection formation involves synchronized nuclear divisions and septum formation and results in a clamp-like morphology at the septum of the main hypha. Clamp connections are found only in



Figure 5 Examples of Basidiomycota diversity. Pucciniomycotina: (**a**) uredinia of *Puccinia iridis*; (**b**) fruiting body of *Phleogena faginea*; (**c**) aecia of *Coleosporium*; (**d**) yeast state of *Symmetrospora oryzicola*. Ustilaginomycotina: (**e**) smut galls of *Ustilago maydis*; (**f**) gall of *Exobasidium*; (**g**) culture of *Moniliella* sp. Agaricomycotina: (**h**) culture of *Wallemia*; (**i**) stinkhorn fruiting body of *Phallus* (photo by Nu Nguyen). (**j**) coral fruiting body of *Clavaria*; (**k**) crust fruiting body of *Amylostereum*; (**l**) club fruiting body of *Clavariadelphis*; (**m**) polypore, conk fruiting body of *Pycnoporus*; (**n**) gilled mushroom fruiting body of *Russula*; (**o**) pored mushroom fruiting body of *Boletus*; (**p**) puffball fruiting body of *Lycoperdon*.

Basidiomycota but are not found in all species or all tissue types (e.g., monokaryon versus dikaryon, vegetative hyphae versus sporocarps, etc.).

Molecular phylogenetic and phylogenomic analyses find greater support for the relationship of Pucciniomycotina as the sister to Ustilaginomycotina and Agaricomycotina than for other arrangements and are consistent with phragmobasidia and repetitive spore germination being symplesiomorphic characters inherited from the MRCA of Basidiomycota. As is also true of Ascomycota, sporocarp formation may have evolved multiple times within Basidiomycota, being rare

but scattered throughout Pucciniomycotina and common among most lineages of Agaricomycotina (e.g., jelly fungi, mushrooms, polypores, etc.). The oldest definitive fossil data for Basidiomycota are septate hyphae with clamp connections from the Pennsylvanian (300 to 360 million years ago) that occur associated with gymnosperms and ferns (173, 174). There also exist fossils of mushrooms from the Cretaceous through the Tertiary (reviewed in reference 16).

Pucciniomycotina

One significant outcome of molecular phylogenetics is the discovery of the true phylogenetic, morphological, and ecological diversity of Pucciniomycotina (101, 175). Currently there are 9 classes and over 8,000 species in which yeast and dimorphic growth forms are common. Basidiocarp formation is rare, but fleshy clavarioid or crust-like forms occur in Pucciniomycetes (e.g., *Eocronartium*, *Septobasidium*), and smaller stilboid and pulvinate basidiocarps are formed in some Atractiellomycetes (e.g., *Phleogena*, *Hobsonia*; Fig. 5b), Agaricostilbomycetes (e.g., *Agaricostilbum*), and Microbotryomycetes (e.g., *Pycnopulvinus*), which presumably represents an independent origin of sporocarps from that of Agaricomycotina. Basidia are almost always phragmobasidia, but unicellular forms (holobasidia) are known. The unifying character of the subphylum is the simple septum, which is morphologically similar to the simple septum of Ascomycota that lacks any septal pore cap. The majority of species are plant-associated as pathogens, endophytes, and phylloplane fungi, but there also exist insect pathogens, orchid mycorrhizae, mycoparasites, and freshwater and marine yeasts. Environmental sequencing studies have revealed unknown Pucciniomycotina diversity in anoxic deep sea habitats, Arctic ice, and other extreme environments including niches characterized by extreme osmotic pressures (reviewed in reference 176). Indeed, the modern concept of Pucciniomycotina comprises fungi that occupy a diversity of ecological niches comparable to Agaricomycotina and Pezizomycotina.

The largest class in Pucciniomycotina is Pucciniomycetes, and the best-studied species are plant pathogens in the order Pucciniales, the "rust" fungi, which collectively attack a wide range of plants from grasses to trees (Fig. 5a and 5c). These fungi exhibit the most complex life cycles among the kingdom Fungi, which include multiple (up to five) spore stages that can occur on more than one host. For example, *Puccinia graminis*, rust of wheat and other grasses, produces monokaryotic spermatia and dikaryotic aeciospores on the alternate host *Berberis*. Aeciospores infect the primary host, *Triticum*, resulting in the production of dikaryotic urediniospores and teliospores. Karyogamy and meiosis occur in teliospores, which overwinter in wheat stubble and germinate in the spring to produce basidia and basidiospores, which reinfect the alternate host. Eradication of the alternate host has been applied as one control method for serious disease caused by rust fungi, the most famous example being the Barberry Eradication Program for stem rust of wheat that was in effect in the United States from 1918 to 1980. This program had a positive impact by reducing the amount of inoculum available for infecting wheat in the United States during its implementation (177). This life cycle of occurring on two hosts is referred to as heteroecious, and the production of all five spore stages is termed macrocyclic. Not all Pucciniales life cycles are this complex, however; some only occur on one host (autoecious), and some have lost certain spore stages (demicyclic and microcyclic). Septobasidiales, also in Pucciniomycetes, represents one of the more divergent ecologies of Pucciniomycotina in that its members parasitize scale insects (100, 178). The fungus parasitizes adults of the insect colony, sterilizing them but not killing them, at least initially. The insect continues to feed on the host plant, providing nutrients to the fungus, which in turn provides shelter to other free-living members of the insect colony.

In addition to Pucciniomycetes, Pucciniomycotina includes eight other classes that exhibit a range of ecologies and morphologies (175). Members of Agaricostilbomycetes were originally described as ascomycetes but are now known to be Pucciniomycotina and include plant saprobes and mycoparasites. Atractiellomycetes display diverse ecological strategies including mycorrhizae of neotropical orchids. Classiculomycetes include hyphal species that associate with leaf litter in freshwater environments but may be capable of mycoparasitism, as well. Cryptomycocolacomycetes are mycoparasites and have been isolated from bark beetle galleries. Cystobasidiomycetes are predominantly mycoparasitic yeasts and include some commonly isolated basidiomycete red yeasts formerly placed in the anamorphic genera *Rhodotorula* and *Sporobolomyces* (Fig. 5d), as well as Cyphobasidiales—a yeast lineage recently identified as comprising a third symbiotic partner in many common lichen species (163).

Microbotryomycetes, the second-largest class, contains primarily plant pathogens and saprobic phylloplane fungi. The plant pathogens include the "anther smuts," *Microbotryum violaceum* and relatives, which were originally classified in Ustilaginomycotina because of the smut-like appearance of the teliospores, a con-

vergent habit of infecting host reproductive tissues. Most orders of the class contain yeast species that are common in environmental sampling and represent a large and poorly characterized diversity (reviewed in reference 179), including the ubiquitous red yeasts of the genera *Rhodotorula*, *Sporobolomyces*, and *Sporidiobolus*. Mixiomycetes are an enigmatic monotypic class that was originally classified in ascomycetes based on the production of a sac-like spore-producing structure, although it appears that these spores are mitotic in origin. *Mixia osmundae* is an intracellular parasite of ferns but has also been detected in environmental sequencing of angiosperms, including bamboo and beach (180). Finally, Tritirachiomycetes are another group that was originally classified as ascomycete molds. No sexual state has been observed for the group. It is isolated from dead plant material and indoor environments and is suspected of being a mycoparasite of ascomycete molds, such as *Penicillium* (181).

Genomic sequencing of Pucciniomycotina is rapidly advancing and includes some of the largest (*Melampsora allii-populina*, 335 Mb; http://genome.jgi.doe.gov/) and some of the smallest (*Mixia*, 13.6 Mb [180]) filamentous fungal genomes sequenced to date. Comparison of pathogenic species of Pucciniomycetes revealed genomic features related to their biotrophic ecology including a large number of SSPs, diminished nitrogen and sulfur assimilation pathways, and expanded families of membrane transporters (182). SSPs along with secreted hydrolytic enzymes and membrane transporters are upregulated *in planta*, consistent with functions in host infection and nutrient acquisition.

Ustilaginomycotina

The subphylum Ustilaginomycotina includes the smut fungi and relatives. The majority of species are plant pathogens, and the term "smut fungi" refers to the black and powdery masses of teliospores produced on the host plant. The smut morphology has been derived convergently in Ustilaginomycotina and Pucciniomycotina (see Microbotryomycetes, Pucciniomycotina) and also occurs in Entorrhizomycetes. This last class contains a small cohort of sedge- and rush-associated smut fungi that cause spore-filled galls to form on host roots. Traditionally placed within Ustilaginomycotina, this group has recently been elevated to phylum status (183), although robust phylogenetic and genomic data are needed to definitively resolve their relationship to other Dikarya. In Entorrhizomycetes, the septal pore is of the dolipore type and lacks a septal pore cap, similar to that of the smut *Tilletia* (Ustilaginomycotina, Exobasidiomycetes [184]).

The cell walls of Ustilaginomycotina are unique in that they contain high proportions of glucose and an absence of xylose, distinguishing them from the rest of the Basidiomycota (185). Their hyphal septa can be swollen near the septal pore, reminiscent of dolipores, and although they lack a septal pore cap, they may possess a membranous pore cap associated with the septal pore (reviewed in 186). They also possess a characteristic host-parasite interaction zone defined by fungal exocytosis of interaction vesicles (179). The genomes of Ustilaginomycotina are some of the smallest among the Basidiomycota, ranging from ~8 Mb and 4,000 genes in animal-associated yeasts of *Malassezia* (187) to 24 Mb and 8,400 genes in phylloplane species of *Tilletiopsis* (http://genome.jgi.doe.gov/programs/fungi/).

The subphylum includes important plant-pathogenic fungi that occur on angiosperms, especially grasses and sedges, with some exceptions. The majority of Ustilaginomycotina species exhibit a dimorphic life cycle that includes a haploid, saprobic yeast phase and a dikaryotic, filamentous biotrophic or pathogenic phase. Yeast phases can be found on numerous plant substrates, and the filamentous phase is initiated by the mating event. Young basidia become thick-walled and darkly pigmented and develop into teliospores. Teliospores germinate promycelia that may be septate (e.g., phragmobasidia of *Ustilago*) or not (e.g., holobasidia of *Tilletia*), depending on the species. The majority of smut fungi produce basidiospores that are capable of repetitive germination, giving rise to secondary spores called sporidia. Sporidia grow and divide as yeasts until mating between two sporidia reconstitutes the filamentous phase.

Ustilaginomycotina consists of four classes: Exobasidiomycetes, Malasseziomycetes, Moniliellomycetes, and Ustilaginomycetes. Most species of Exobasidiomycetes produce holobasidia, and teliospores may be present or absent according to group. *Exobasidium* species (Exobasidiales) are biotrophic primarily on members of Ericaceae (Fig. 5f); they lack a teliospore and sporulate by producing long holobasidia through stomata or from the epidermis (188). *Tilletia* (Tilletiales) is a particularly notorious plant pathogen responsible for karnal bunt of wheat and diseases of other grasses including barley and rice. It is not known to be dimorphic, and basidiospores often conjugate on the basidium, directly giving rise to filamentous dikaryotic growth. Infections can cause large losses in production, and contamination of grains with *Tilletia* teliospores has had a profound impact on agricultural trade due to plant quarantine regulations (189).

Malasseziomycetes are lipophilic yeasts. They are unusual among Ustilaginomycotina in that they are not

plant associated but are found on the skins of mammals including humans. *Malassezia* species are sometimes referred to as dandruff fungi, and the genomes of multiple species have been sequenced with the first, *M. globosa*, sequenced by Procter & Gamble, the manufacturers of Head & Shoulders shampoo (187). Comparative genomics of multiple species have revealed that these fungi are incapable of fatty acid biosynthesis and are dependent upon a lipid-rich diet. To compensate for its inability to synthesize fatty acids, *Malassezia* possesses numerous secreted lipases and other hydrolases for securing host lipids (187, 190). And while *Malassezia* species are only known as asexual yeasts, their genomes do possess the genes necessary for mating. Recently, environmental sampling studies have identified *Malassezia* as a commonly encountered marine fungus, although its function in these ecosystems is unknown (191). Moniliellomycetes also consists of lipophilic yeasts (Fig. 5g) that were traditionally classified with other fungi now placed in Agaricomycotina. However, recent molecular phylogenies suggest it is a distinct class-level taxon in Ustilaginomycotina (192). Most species are known from industrial settings, foods, fats, oils, or substrates with low water activity. The septal pore apparatus of *Moniliella oedocephalis* is of the dolipore type but without pore caps (193), which may further support the current placement within Ustilaginomycotina.

Most Ustilaginomycetes species are dimorphic plant pathogens and usually produce teliospores in the reproductive organs of their host. *Ustilago maydis*, corn smut, is the best-studied species due to corn's importance in agriculture as food and biofuel feedstock. The fungus infects the plant through the developing ovaries but can colonize all parts of the host, resulting in chlorosis, anthocyanin formation, and reduced growth. The production of teliospores occurs in kernels of corn that have been infected by the fungus and coopted for spore production. The result is that a kernel is transformed into a large gasteroid "tumor" or gall filled with teliospores (Fig. 5e). Teliospores are darkly pigmented and produce four-celled phragmobasidia with basidiospores. Basidiospores divide by budding, producing a haploid and saprobic yeast phase. Two yeasts of opposite mating types conjugate to initiate the dikaryotic and pathogenic filamentous phase of the life cycle. Although the pathogens can be quite destructive on grains, some, such as the galls of *U. maydis*, which are a delicacy in Mesoamerica called huitlacoche, are edible and have gained increasing popularity in adventure eating in other parts of the world (194).

Genome analyses of *Ustilago* revealed a small genome of approximately 20 Mb encoding less than 7,000 genes with no known pathogenicity factors (195). Rather, SSPs, which exist in small gene families, were demonstrated to function in pathogenicity. These SSPs are coregulated and expressed in infected tissue, and SSP mutants exhibited a range of phenotypes from reduced to increased virulence. The discovery of SSPs and demonstration of their role in infection and pathogenicity have since transformed the study of biotrophic fungi including pathogens and beneficial symbionts (196).

Agaricomycotina

Agaricomycotina is currently divided into four classes: Wallemiomycetes, Tremellomycetes, Dacrymycetes, and Agaricomycetes. Wallemiomycetes is sister to the remainder of Agaricomycotina and consists of ascomycete-like molds that do not produce sporocarps and are capable of withstanding conditions of high osmotic stress (Fig. 5h) (168). Tremellomycetes and Dacrymycetes include the majority of fungi with gelatinous sporocarps, or jelly fungi, while Agaricomycetes includes some jelly fungi and the remainder of fleshy, sporocarp-producing species (e.g., mushrooms). Tremellomycetes is sister to Dacrymycetes and Agaricomycetes and contains three orders: Cystofilobasidiales, Filobasidiales, and Tremellales. Cystofilobasidiales and Filobasidiales comprise species that are yeasts or are dimorphic, with sporocarp production being unknown. Tremellales includes many of the well-known jelly fungi (e.g., *Tremella mesenterica*, witches butter) and important human pathogens such as *Cryptococcus*. Species of *Tremella* fruit from wood and produce phragmobasidia with longitudinal septations dividing the basidium into four equal compartments with long, slender sterigmata. Many species in the class are either known or suspected to be mycoparasites and likely play a role in parasitizing wood-inhabiting fungi. *Cryptococcus* is a common inhabitant of soils, plant material (e.g., bark), and bird guano. It grows primarily as yeast in host tissue but is dimorphic, producing holobasidia with long chains of basidiospores. *Cryptococcus neoformans* is an important human pathogen, especially of people with compromised immune systems, but *Cryptococcus gattii* is known to infect immunocompetent people who were otherwise healthy (197). Dacrymycetes includes a single order, Dacrymycetales, and is the sister group to Agaricomycetes. Species of this class produce Y-shaped basidia in gelatinous sporocarps that fruit from wood. Dacrymycetales are characterized as brown rot fungi in which wood decay involves the breakdown of cellulose and hemicellose, but not lignin.

Agaricomycetes comprise the majority of fleshy, sporocarp-producing basidiomycetes. The diversity of fruiting bodies includes mushrooms and boletes, polypores and conks, crusts, coral fungi, puffballs and truffle-like fungi, and stinkhorns. Historically, these fungi were classified as hymenomycetes and gasteromycetes in a system attributed to Elias Fries in what is frequently referred to as the Friesian system (198). Hymenomycetes (hymenial fungi) produce basidia and basidiospores on a basidia-producing tissue called the hymenium that is exposed to the environment and forcibly eject their basidiospores. These fungi include the mushrooms, boletes, corals, crusts, polypores, and conks. Gasteromycetes (stomach fungi) produce basidia in an enclosed region of the sporocarp, the gleba, and do not forcibly eject their basidiospores. Spores may be dispersed by wind and rain, as in the puffballs (Fig. 5p), or by animal mycophagy or phoresis, as in truffles and stinkhorns (Fig. 5i), respectively. We now understand that hymenomycetes and gasteromycetes are artificial taxa and that these forms are intermixed through the phylogeny of Agaricomycetes. Molecular phylogenetic analyses resolve the evolutionary transition in spore dispersal from forcibly discharged to passively discharged basidiospores, with gasteromycetes being derived from hymenomycetes on multiple occasions (199, 200). Moreover, most sporocarp morphologies have been derived multiple times, including the mushroom morphology characterized by a stipe, a gilled or pored hymenium, and a cap (Fig. 5n). The evolutionary plasticity of the sporocarp is likely a result of strong evolutionary selection pressures on production and dispersal of basidiospores.

The modern understanding of Agaricomycetes evolution is the result of numerous studies of molecular phylogenetics (e.g., 201) and evolutionary genomics (e.g., 17, 202), with the outcome being significant revisions to the premolecular taxonomy of the class. Currently, there are 21 orders of Agaricomycetes, with 6 orders classified in the subclass Agaricomycetidae, 4 in Phallomycetidae, and the remaining 11 treated as *incertae sedis*. Auriculariales, Sebacinales, and Cantharellales represent some of the first orders to diverge since the MRCA of Agaricomycetes, but the branching order of these taxa is unresolved. Auriculariales and Sebacinales include species that produce gelatinous sporocarps and, in some species, phragmobasidia, providing further evidence that these traits are ancestral for Agaricomycetes. Cantharellales is best known for prized edible forest mushrooms of *Cantharellus*, but the order includes numerous morphologies including toothed fungi (e.g., *Hydnum*), coral fungi (e.g., *Clavulina*), and crusts (e.g., *Botryosphaeria*). Phallomycetidae is one of the more morphologically diverse clades and contains four orders: Phallales (the stinkhorns; Fig. 5i), Geastrales (earthstars), Gomphales (coral fungi of *Ramaria* and cantherelloid fungi of *Gomphus*; Fig. 5j and 5l), and Hysterangiales (basidiomycete truffles). Agaricomycetidae contains many of the best-known mushroom-forming taxa, including Agaricales and Boletales, but the modern definition of these orders includes numerous other morphologies. For example, Agaricales also includes bird's nests of *Nidularia*, puffballs of *Lycoperdon* (Fig. 5p), coral fungi of *Clavaria* (Fig. 5j), truffles of *Hydnangium*, and oyster mushrooms of *Pleurotus*. Likewise, in addition to the pored mushrooms of boletes (Fig. 5o), Boletales includes truffles of *Rhizopogon*, earthballs of *Scleroderma*, and resupinate fungi of *Serpula*.

Other orders of Agaricomycetidae include crust-forming fungi (Atheliales and Jaapiales) and clavarioid basidiolichens (Lepidostromatales).

The remaining orders of Agaricomycetes mostly include nongilled fungi that were once accommodated in the concept of Aphyllophorales, an old order name meaning "without gills" (203). These include the polypores (Fig. 5m), conks, and shelf fungi (Gloeophyllales, Hymenochaetales, Polyporales, Stereopsidales, Thelephorales, Trechisporales) and the crust or parchment fungi (Corticiales). Perhaps the most remarkable order of Agaricomycetes is Russulales. This order of fungi contains all known major morphologies of fleshy sporocarps, including mushrooms (*Russula* and *Lactarius*), polypores (*Bondarzewia*), tooth fungi (*Auriscaplium*), crusts (*Aleurodiscus*), coral fungi (*Clavicorona*), and truffles (*Gymnomyces*), and it demonstrates the "tricks" that evolution has played on fungal taxonomists. Interestingly, most of the fungi of Russulales form basidiospores with wall ornamentation that stain blue to black in iodine solution, the positive amyloid reaction. For a more complete review of Agaricomycetes systematics and taxonomy see Hibbett et al. (204).

Agaricomycetes are dominant forest fungi, where they function as ectomycorrhizal symbionts, tree pathogens, and agents of wood and litter decay. Comparative and evolutionary genomics have provided significant insight into the evolution of these ecologies. Saprobic, or plant-decomposing, Agaricomycetes appear to be the ancestral ecology, with symbiotic lifestyles such as ectomycorrhizae being more derived. Wood-decay Agaricomycetes are categorized into two major groups, white rot and brown rot, although comparative genomic analyses of wood-decay species reveal the inadequacy of a simple two-category system (205). White rot fungi

are capable of breaking down cellulose, hemicellulose, and lignin at roughly equal rates. Brown rot fungi do not break down lignin to any appreciable degree. The ability to efficiently break down lignin is attributed to major innovations of wood- and lignin-degrading enzymes, the fungal class II peroxidases (PODs), in the common ancestor of Agaricomycetes (17). Phylogenomic analyses support a diversification of PODs in the late Permian, leading to the hypothesis that the rise of wood-decay fungi and the diversification of their enzymatic machinery resulted in dramatic decreases in lignified coal deposits at the end of the Permian (17). Brown rot fungi have been derived multiple times through the loss of fungal PODs and the ability to degrade lignin. Major white rot orders include Auriculariales, Hymenochaetales, Corticiales, Polyporales, Russulales, and Agaricales, while brown rot has evolved independently in Gloeophyllales, Polyporales, and Boletales.

Ectomycorrhizae ("outer" + "fungus" + "root") are fungi that form symbioses with forest trees, especially species of Pinaceae and Fagaceae. They associate with the fine roots of the plant, but they do not penetrate the cells of the plant—thus the term "ecto." Rather, they form a sheath around the cortex cells of the fine roots. The symbiosis is based on an exchange of common goods, with the fungus providing water and mineral nutrients (e.g., phosphorus, nitrogen, etc.) to the plant and the plant providing sugars (e.g., glucose) to the fungus. Ectomycorrhizae have been derived numerous times during the evolution of Agaricomycetes, including within Agaricales, Boletales, Russulales, Hymenochaetales, and Cantharellales, and from both white rot and brown rot ancestors. Comparative genomics have revealed some consistent themes that allow a fungus to adopt an ectomycorrhizal lifestyle (197). First, these fungi lose much of the enzymatic machinery (carbohydrate active enzymes), especially cellulases, associated with the breakdown of plant cell walls. Second, they have evolved SSPs that interact with the plant's host defense system. Together these attributes allow the fungus to colonize plant roots and not be identified by the plant as a hostile intruder. Also, many ectomycorrhizal fungi exhibit significant genome expansions, but not in gene content. These genome expansions are due to an increase in the abundance of transposable elements. The function of these transposable elements is unknown, but it hypothesized that they may promote genomic adaptations.

SUMMARY

Molecular and genomic analyses of the fungal tree of life have shown that numerous morphologies emphasized in premolecular classification (e.g., zoospores, zygospores, fruiting body morphology, etc.) are not diagnostic of monophyletic groups. Rather, the taxa that possess these traits experienced more complicated patterns of diversification and are frequently paraphyletic. The first three lineages to diverge since the LUCA of kingdom Fungi comprise mostly zoosporic fungi, Cryptomycota, Blastocladiomycota, and Chytridiomycota. While losses of flagellum are known within the zoosporic clades and presumably in the MRCA of the remaining phyla of nonflagellated fungi, the definitive number and placement of flagellum losses are currently disputed due to insufficient taxon and data sampling (e.g., the genus *Olpidium*). Zygomycete fungi comprise two separate phyla of nonflagellated fungi: Zoopagomycota and Mucoromycota. They display differences in host and substrate associations—Zoopagomycota with animals and fungi, Mucoromycota with plants and plant substrates—and may represent morphologies and lifestyles of the first terrestrial fungi. Ascomycota and Basidiomycota form the Dikarya and possess the most derived traits, representing the apex of morphological complexity among the fungi.

Our understanding of fungal evolution has been significantly influenced by molecular and genome technologies, in both unraveling the aforementioned phylogenetic relationships and illuminating processes of adaptation and diversification. The sequencing of fungal genomes is quickly becoming routine, representing the starting point for an increasing number and types of studies. This is resulting in a rapid increase in the number of species that can be incorporated into genome-scale phylogenies, as evidenced by MycoCosm, with more than 800 fungal genomes (http://genome.jgi.doe.gov/fungi/). Continued taxon sampling should exploit existing resources of biological culture collections to continue populating the fungal tree of life with genome data from unsampled species and lineages. Doing so will not only incorporate the global collecting effort of mycologists across generations, but it will also add significant value to existing isolates, making them more amenable to inclusion in a wider range of research. The next wave of genome sampling must also incorporate a greater diversity of fungi that cannot be maintained in culture. This will require sampling of sporocarps and spores and will in most cases represent metagenomes with resident populations of bacteria and other eukaryotes. While this approach is more computationally intensive, extraction of phylogenetically informative markers is tractable (139). Furthermore, significant progress has been made in grouping sequences by organism to achieve accurate assemblies

of the composite of individuals within a metagenome (206, 207), and increased sporocarp sampling will provide more data examples for refinement of algorithms and computational pipelines. Continued advancements in genome-scale phylogenies of fungi are not completely dependent upon sampling alone, however. Advancements in models of evolution are needed to understand conflict among data partitions more accurately and better discern between complicated processes such as incomplete lineage sorting and insufficient phylogenetic signal (6).

All phylogenetic analyses are inherently biased by what has been called the "invisible dimension of fungal diversity" (208). Environmental sampling of ecosystems and niches is consistent with the existence of numerous unknown higher-level lineages (209). While some of these have been brought into culture and expanded our knowledge of sparsely populated lineages (e.g., 25, 111), an urgent need to culture more of these fungi exists, resulting in biological resources necessary for functional analyses. Currently, the incorporation of unknown fungi into phylogenetic analyses is limited to a few loci of limited phylogenetic informativeness that can be obtained through amplicon-based sampling approaches. The holy grail of metagenomic sampling would be the sequencing of, and access to, the complete genomes of all or most of the organisms in a sample. Such data would have considerably more explanatory power within and beyond phylogenetics (e.g., ecological genomics, EvoDevo, etc.).

Finally, a greater need for integration of other types of data (e.g., fossils, ecology, physiology, etc.) with genomic data and genome-scale phylogenies exists. Fossils of fungi are more rarely reported than those of plants and animals, but the number of researchers studying paleomycology is as much of a limiting factor as the fossilization rate of fungi. As more fossils are described, greater effort should be given to integration of these data in phylogenies and the development of relaxed clock methods so that major events in fungal evolution can be incorporated more accurately into other biological and geological events of the Earth's history. Fungal ecology and ecological genomics are rapidly growing, and significant advancements have been made in understanding evolutionary transitions to mycorrhizal symbioses, wood decay, and plant pathogenesis. Systematic integration of metagenomic (e.g., short sequence read libraries, metatranscriptomes, etc.) with genome-derived phylogenies is needed, however, to better understand the distribution of fungi and fungal metabolic traits across ecosystems and ecological niches.

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Six Key Traits of Fungi: Their Evolutionary Origins and Genetic Bases

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INTRODUCTION

The fungal lineage is one of the three large eukaryotic lineages that dominate terrestrial ecosystems. They share a common ancestor with animals in the eukaryotic supergroup Opisthokonta and have a deeper common ancestry with plants, yet several phenotypes such as morphological, physiological, or nutritional traits make them unique among all living organisms. This article provides an overview of some of the most important fungal traits, how they evolve, and what major genes and gene families contribute to their development. The traits highlighted here represent just a sample of the characteristics that have evolved in fungi, including polarized multicellular growth, fruiting body development, dimorphism, secondary metabolism, wood decay, and mycorrhizae. However, a great deal of other important traits also underlie the evolution of the taxonomically and phenotypically hyperdiverse fungal kingdom, which could fill up a volume on its own. After reviewing the evolution of these six well-studied traits in fungi, we discuss how the recurrent evolution of phenotypic similarity, that is, convergent evolution in the broad sense, has shaped their phylogenetic distribution in extant species.

THE EVOLUTION AND DE-EVOLUTION OF MULTICELLULARITY

Multicellular life dominates most ecosystems on Earth, and its evolution is considered to be one of the major transitions in the history of life. Multicellularity confers several competitive advantages. Among others, it allows the division of labor between cells, increased size and complexity, a longer life span, or an advantage in avoiding predation. It has been estimated that multicellularity evolved in some form 25 to 30 times both in the prokaryotes and the eukaryotes, but most of those lineages are simple aggregates or colonies of cells that show little phenotypic or functional differentiation. Fungi represent one of the few lineages that have achieved higher levels of multicellular complexity. Like animals and plants, fungi evolved integrated multicellular structures that enable them to adapt to diverse ecological niches. However, unlike animals and plants, fungi evolved complex multicellularity through filamentous intermediate stages. Fungal filaments (hyphae), unlike filamentous forms in other lineages, develop by polarized apical growth, and individual cells are divided from the elongating hypha by developing cross-walls (septa), a unique solution for filamentous growth seen mostly in higher fungi (1). Hyphae have been hypothesized to have emerged through gradual elongation of substrate-anchoring rhizoids of ancestral unicellular fungi such as chytrids (2).

Similar, apically growing hyphae have evolved in the Oomycetes (Stramenopila), but oomycete hyphae are not septate, allowing the transport of compounds and organelles along the hyphae. Such aseptate hyphae are a multinucleate, coenocytic architecture, similar to hyphae of early-diverging fungal groups (e.g., Mucoromycota). Within fungi, hyphae evolved convergently

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in multiple groups (Fig. 1A,B), one of them comprising most of the well-known filamentous fungi. Other hyphal groups include the Monoblepharidomycetes, an early-diverging lineage of aquatic fungi in which several species have switched to filamentous growth (3), and certain chytrid-like fungi.

Fungal mycelia—intricate branching networks of hyphae—are adapted to efficiently explore the available space in the substrate. Foraging for nutrients may have driven the emergence of multicellularity in fungi, although transition to terrestrial habitats and exploitation of prokaryotic biofilms have also been proposed as the necessary selection pressures for their emergence (4). The first multicellular hyphae evolved in the common ancestor of the Zoopagomycota, Mucoromycota, and the Dikarya and were probably similar to those of extant Mucoromycota mucoralean fungi such as the bread mold *Rhizopus stolonifer*. Early filamentous species have relatively simple, little-differentiated mycelia of tube-like cells that are not (or are minimally) compartmentalized by septa. More derived fungi evolved various solutions to block the free diffusion of materials in hyphae, which would also minimize the risk of the free passage of external invaders (5). Specialized structures around septa, including Woronin bodies and the dolipore septum among others, serve to regulate transport between cells in the hyphae of Asco- and Basidiomycota, respectively, although exceptions to this rule exist. *Neolecta vitellina*, a basal ascomycete (Taphrinomycotina), produces crystalline bodies analogous to Woronin bodies but which are of vacuolar origin and only loosely bound to the membrane (6). Although structurally different, all three solutions provide the means to finely coordinate the transport of goods and to block the diffusion of organelles from one cell to the other.

Polarized Growth and the Underlying Cellular Components

Polarized hyphal growth of fungi is a unique innovation in the history of life, with similar solutions seen only in a few other groups such as Oomycota and the pollen tubes of seed plants and axons of some neurons. The orchestration of hyphal growth involves several cellular pathways and entailed the evolution of several novel traits, including the establishment of polarity, apical extension, the coordinated transport of material to the sites of active growth, the establishment of branching, and septation patterns. In hyphae, growth occurs in the apical zone, coincident with a strong gradient of cell wall material deposition toward the tip. Apical growth is achieved by the expansion of the hyphal tip, which is predominantly driven by turgor pressure within the cell that, in turn, is regulated by the osmotic mitogen-activated protein kinase cascade (7). Cell wall materials are transported by secretory vesicles to the growing tip along cytoskeletal structures (7). One of the core signal transduction pathways connected to polarity establishment is the Ras/Rho GTPase pathway described in *Saccharomyces cerevisiae*, which is also conserved in filamentous fungi. This pathway is responsible for polarity establishment and maintenance by the formation of a stable polarity axis that specifies the site of germ tube emergence. This positional information is generated by the recruitment of cell polarity proteins (Bud4, Axl2, swoC in *Aspergillus nidulans*) at the site of polarized growth (8) and then is transduced via regulatory Rho GTPases (RacA *A. nidulans*, CDC42 *S. cerevisiae*) toward the morphogenetic machinery, which is the main organizer of tipward secretion, cell wall expansion, and tip growth (1, 9).

The key contributor to the hyphal morphogenetic machinery is the Spitzenkörper (10). It is hypothesized to serve as a vesicular distributor between the microtubular vesicular transport system and the actin patches accumulated at the apex, and therefore it is responsible for the growth directionality of hyphae. These vesicles transport structural and landmark proteins, proteins that facilitate membrane docking and fusion, different enzymes (chitin synthase), and Ca^{2+}, components that are essential for hypha elongation. The microtubule network connected to the Spitzenkörper has an indirect role in polarity maintenance and hyphal growth and is responsible for the long-range vesicular transport to the apex (11).

In addition to the microtubular network, the actin cytoskeleton is also connected to the Spitzenkörper. It has a crucial role in taking vesicles delivered to the apex via myosin-based transport and guiding them to polarization/secretion sites serving as a bridge between the Spitzenkörper and the extension site of the hyphal apex. The actin cytoskeleton is regulated by different polarity-establishing and -maintaining factors: the polarisome and the Arp2/3 protein complex. Both of these complexes have a role in actin polarization; the polarisome is responsible for the organization of the actin cytoskeleton to the expansion site by formin-dependent actin polarization (SepA and MesA in *A. nidulans*; Spa2, Bud6, and Bni1 in *Neurospora crassa*). The Arp2/3 complex in turn controls the building of cortical actin patches at the apex which have a crucial role in endocytosis (12).

Precise coordination of vesicle delivery to the expansion site is essential for polarized growth. The key

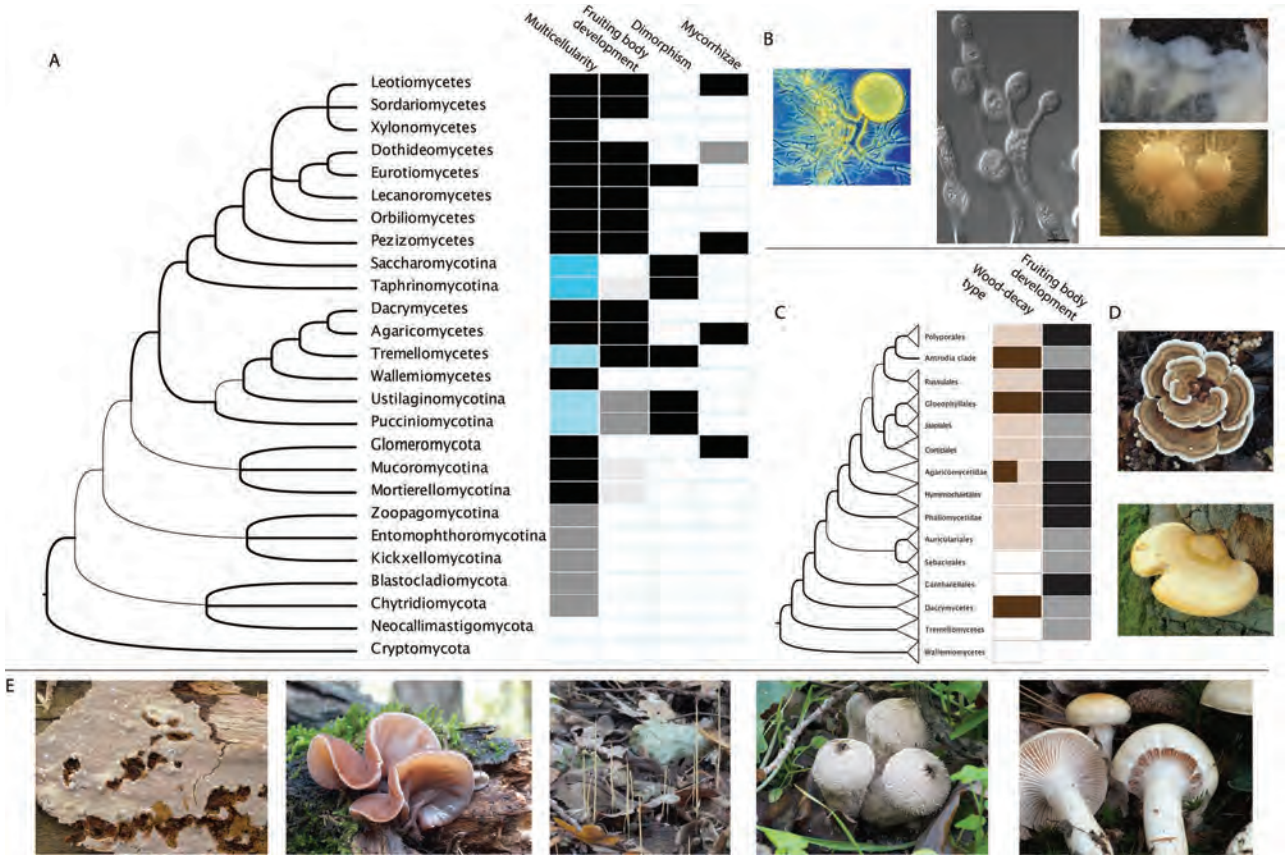


Figure 1 Overview of the phylogenetic distribution of the traits presented in this article. (**A**) Phylogeny of the major fungal groups and the phylogenetic distribution of the traits discussed in this article. Tree modified and redrawn from MycoCosm (http://genome.jgi.doe.gov/programs/fungi/index.jsf). Thick branches denote well-known relationships, while thin branches mark uncertainties in our understanding of fungal relationships. Presence or absence of a given trait is highlighted by black or white shading, respectively. Gray shading denotes rare or not fully developed states of the given trait, while blue denotes the secondary partial loss of multicellular growth in yeasts. (**B**) The evolution of hyphae from unicellular ancestors (left, *Spizellomyces punctatus*; photo courtesy of Don Barr, http://www.bsu.edu/classes/ruch/msa/barr/4-15.jpg). Hyphae evolved in several groups, including the Monoblepharidomycetes (middle, *Monoblepharella* sp.; [from reference 3]) and the crown fungi (top right, *Psathyrella spadiceogrisea* mycelium growing on sawdust). Also shown are hyphal and yeast-like colonies of the dimorphic fungus *Aureobasidium pullulans* (bottom right). (**C**) Detailed view of the major orders, their wood-rotting characteristics, and the distribution of agaricoid fruiting body morphologies of the Agaricomycetes. Brown and beige represent brown and white rot lineages, respectively, whereas empty rectangles denote groups that do not degrade wood (Wallemiomycetes, Tremellomycetes) or cause an uncharacterized type of wood decay or mycorrhiza (Sebacinales and Cantharellales). (**D**) Examples of typical white (*Trametes versicolor*, top) and brown (*Piptoporus quercinus*, bottom) rot Basidiomycota. (**E**) Examples of the diversity of fruiting body morphologies in the Agaricomycetes, including from left to right, resupinate (*Cylindrobasidium evolvens*); auricularioid, jelly fungi (*Auricularia auricula-judae*); club and coral fungi (*Typhula phacorrhiza*); puffballs (*Lycoperdon perlatum*); and agaricoid morphologies (*Hygrophorus* sp). Photos: L. Nagy unless stated otherwise.

regulators of this process are the Coatomer complex that mediates vesicle formation (SEC proteins, Sec21 in *N. crassa*), the exocyst complex (SEC proteins, Exo-70, and Exo-84 in *S. cerevisiae*; RabA/B in *A. nidulans*), which is a conserved multiprotein complex with a role in vesicle targeting and vesicle distribution from the Spitzenkörper, and finally, the SNARE complex (nsyn1 and nsyn2 SNAREs in *N. crassa*), which facilitates vesicle fusion with specific membrane types (13).

The rapid rate of hyphal extension requires a large amount of cell wall components deposited at the extension site. According to the steady state theory of hyphal wall growth, the plastic wall material (chitin, 1-3beta glucan) has to be synthesized and delivered continuously to the apex to elongate the hypha. After deposition, cross-linking mechanisms rigidify the proto cell wall material, and only the hyphal apex is plastic enough to grow continuously. This process is coordinated by various cell wall-synthesizing enzymes: glucanases and different types of chitin synthases (chsB/D/E in *A. nidulans* and chs1 in *N. crassa*).

Along with the elongated morphology, septation and branching patterns are also characteristic morphological features of filamentous fungi. Mitotic signals and cortical markers are the inducers of septation, and the morphogenetic machinery behind this process is composed of actin cytoskeleton, septins (aspA-E in *A. nidulans*), and formins (sepA/D/G/H) (14). Septation and branching are two morphogenetically connected events. In septum-forming fungi, the branching event occurs just after septation and starts with the generation of a new polarity axis, followed by the recruitment of the morphogenetic machinery to induce branch emergence. In *N. crassa*, the characteristic pod proteins (pod4/5/8) are required for branch formation, but interestingly, they have no obvious role in the morphogenesis of primary hyphae (8).

The De-Evolution of the Multicellular Life Stage

Many fungal groups seem to have secondarily lost multicellularity. These are collectively known as yeasts, and they are some of the best-studied and economically most important eukaryotic organisms such as S. *cerevisiae* and *Schizosaccharomyces pombe*. The term "yeast" covers a polyphyletic assemblage of phenotypically similar fungi, many of which are only distantly related to each other. It usually refers to species that are unicellular through most of their life cycle and divide through budding or fission. Such species evolved in many major groups, including the Asco- and Basidiomycota and certain Mucoromycota, and can be found in large clades such as the Saccharomycotina or derived as smaller groups within diverse clades of filamentous fungi. The most widely known ascomycete yeasts include the genera *Saccharomyces*, *Candida*, *Pichia*, and *Yarrowia* in the Saccharomycotina subphylum; *Schizosaccharomyces* in the Taphrinomycotina; as well as several species in phylogenetically dispersed clades (for details see reference 15). Basidiomycete yeasts occur in two large clades: the Pucciniomycotina (e.g., *Rhodotorula* and *Sporobolomyces*) and the Tremellomycetes (e.g., *Cryptococcus* and *Xanthophyllomyces* [formerly *Phaffia*]). Yeasts evolved from multicellular filamentous ancestors; the independent origin of similar cellular morphologies in diverse groups is a fascinating example of convergent evolution and probably reflects adaptation to similar niches. With their unicellular habit, morphology, and limited dispersal abilities, yeasts are adapted to liquid environments and to the absorption of simple sugars, in contrast to filamentous fungi, which can forage for nutrients over larger distances and drier environments. In nature, yeasts are mostly found in aquatic habitats or moist, sugar-rich environments such as fruits, floral nectars, and other plant exudates or as members of the intestinal microbial flora of animals.

Most people view yeasts as unicellular eukaryotes that have greatly contributed to our understanding of several conserved cellular processes of eukaryotes. Because of this, yeasts are often viewed as the de-evolution of multicellularity in fungi. However, most yeasts, even those that grow as single-celled organisms during most of their life cycle, are able to switch to filamentous growth as a response to changing environmental conditions (for details see the section on dimorphic fungi). Some of them produce only pseudohyphae, elongate to cylindrical cells that are more similar to elongated yeast cells than to real hyphae. However, many species can grow typical hyphae too. Thus, most yeasts are not unicellular in the traditional sense (16).

The mostly unicellular Saccharomycotina group contains *Eremothecium gossypii* (*Ashbya gossypii*), a species that produces elongate, unseptate hypha-like cells that resemble the true hyphae of filamentous fungi. The phylogenetic position of *E. gossypii* within yeasts suggests that its filamentous cells represent a step toward the re-evolution of hyphal growth, although molecular studies suggest that the filaments of *E. gossypii* are more similar to elongated yeast cells than to true hyphae (9).

Yeasts have small, streamlined genomes compared to filamentous fungi, containing 6,000 to 8,000 genes, on average. Their evolution from filamentous ancestors

represents a classic example of convergent evolution and has been marked by extensive gene losses, on average, 3,000 to 5,000 lost genes per lineage, which affected a wide array of genes related to complex polysaccharide metabolism such as genes coding for plant cell wall-degrading enzymes (PCWDEs), secondary metabolism, oxidoreductases, cyclophilins, or hydrophobins (17). Their highly streamlined genomes, however, contain many genes required for hyphal morphogenesis, suggesting a close relationship between multicellular hyphal growth and yeast divisions and/or pseudohyphal growth. Recent results suggest that the convergent evolution of yeast-like fungi is a result of parallel deployment of dormant developmental potentials, rather than the result of the independent evolution of similar genetic toolkits. Convergent diversification of conserved (Zn-cluster) transcription factor families might have led to the potential for yeast-like growth to become dominant in the life cycle at the cost of partial or complete loss of hyphal growth (17). It has also been shown that the genes required for yeast-like growth, including chitin and glucan synthases, as well as chitinases and glucanases involved in separating mother and daughter cells, evolved early in fungi and are shared by most—both filamentous and yeast-like—fungi, which raises the question of what function these genes are responsible for in nonyeast fungi. Because of the similarity between yeast budding and fission and conidium and arthrospore generation, it is tempting to speculate that the cradle for yeast-like growth could have been the morphogenetic machinery of asexual reproduction, although this needs to be tested.

FUNGAL DIMORPHISM

Dimorphic fungal species have the ability to reversibly switch between hyphal and yeast or yeast-like morphologies in response to different stimuli (Fig. 1B), particularly shifts in environmental conditions. Dimorphism is a frequent characteristic of pathogenic fungal species of plants, insects, and humans and other mammalian hosts, and it contributes to their capacity to cause tens of millions of infections annually worldwide. Generally, in dimorphic pathogens of insects and humans and other mammals, the yeast phase is considered to be more pathogenic because this form is able to spread throughout the body, resulting in systemic infection. The role of filamentous growth is also fundamental: besides promoting host tissue adhesion, this form also contributes to transmission to new hosts, maintains survival in the environment, and is required for mating and thus for genetic diversity.

In phytopathogenic fungi, the role of yeast forms in pathogenicity is species specific. Usually it is the mycelial phase that infects plant tissues. In both morphological states one can distinguish between yeast-phase-specific and hyphal-phase-specific gene expressions, while most of the housekeeping genes remain constitutively expressed regardless of the cell type. The expression of phase-specific genes depends on the morphotype and might be associated with virulence.

The six primary pathogens in the Ascomycota that are traditionally classified as dimorphic fungi and cause systemic disease in humans are *Blastomyces dermatitidis* (blastomycosis), *Coccidioides immitis* (coccidioidomycosis, or valley fever), *Histoplasma capsulatum* (histoplasmosis), *Paracoccidioides brasiliensis* (paracoccidioidomycosis), *Sporothrix schenckii* (sporotrichosis), and *Penicillium marneffei* (penicilliosis) (18).

Molecular Mechanisms of Switching Between Yeast and Hyphal Stages

A common mnemonic used for thermally dimorphic fungi is "mold in the cold, yeast (yeast-like) in the heat." In general, the majority of these species are environmental saprobes of the soil, water, etc. and grow as molds at 20 to 25°C. Once the environmental milieu is disrupted and the conidia (e.g., spores) or hyphal fragments are inhaled by or inoculated into humans or other mammalian hosts, dimorphic transition from hyphal growth to yeast or spherule (*Coccidioides* spp.) occurs at 37°C (19, 20).

Following uptake by alveolar macrophages, a large number of fungal transcripts appear that promote adaptation to the new restrictive environment (21). These transcripts induce hyphae to yeast dimorphic transition, cell wall rearrangement, sporulation, and the expression of other possible virulence-effecting genes that alter metabolic pathways. All are required for successful immune evasion, intracellular survival, and systemic dissemination (18). Dimorphic transition is mainly temperature dependent, although other nonthermal signals such as increased CO_2 tension, oxidative stress, and mammalian hormones such as estradiol or L-DOPA could contribute (22–24).

Inside the host, the yeast-like phase is fundamental for successful invasion. During the transition, besides changes in cell size and shape, a large number of yeast-phase- or spherule-phase-specific genes are activated, resulting in cell wall reassembly and alterations in certain signaling pathways (18, 20). For example, β-glucans are one of the most relevant pathogen-associated molecular patterns in the fungal cell wall that are recognized quickly by the host immune cells and enhance efficient

clearance (25). However, in the yeast phase of certain dimorphic fungi, the amount of α-glucan in the cell wall is elevated, which may conceal the immune recognizable β-glucans from the host (26, 27).

Because pathogenicity of these fungi is possibly a consequence of adaptation to the host environment, most signaling pathways associated with virulence are suggested to be sensors of environmental stress. These pathways can include, for example, the protein kinase C cell wall integrity, the cyclic AMP protein kinase A (PKA), calcium-calcineurin, and other two-component pathways. The two main intracellular signaling pathways associated with morphology transition are the cyclic AMP-PKA and the mitogen-activated protein kinase (MAPK) cascade pathways (22). Detection of changes in the amount of the available carbon or nitrogen sources leads to elevated cyclic AMP levels intracellularly, which stimulates PKA. PKA then activates the transcription of factors needed for the dimorphic switch. MAPK cascades consist of several kinases responding to various environmental stimuli and induce a wide variety of response mechanisms. Such kinases include mainly serine, threonine, and tyrosine kinases but also kinases that phosphorylate histidine residues of proteins. A hybrid dimorphism-regulating histidine kinase (*DRK1*) in *B. dermatitidis* was identified and considered to be a global regulator of morphology switch (18). *DRK1* is highly conserved among three dimorphic fungi species and plays a key role in sporulation, hyphae-to-yeast transition, cell wall integrity, and virulence.

Recent studies and observations cumulatively suggest that mammal-infecting dimorphic fungi have evolved several strategies to evade the host immune responses. Such strategies include masking their pathogen-associated danger signals, altering fungal signaling pathways, and promoting intracellular survival. An additional example is the oxidative stress-evading strategy of *H. capsulatum* and *P. brasiliensis* (28–30). Both species evolved their own nitric oxide reductase and several catalase enzymes to overcome the effect of reactive oxygen and nitrogen species produced by host cells.

Dimorphism Is Widespread among Fungi

Another mnemonic that refers to dimorphic fungal pathogens is "Body heat probably changes shape." This phrase allows the inclusion of certain *Candida* spp. in the group of dimorphic species because of the ability of switching morphology in the opposite direction, from yeast to filamentous growth (Fig. 1A). Of these species, *Candida albicans* is known as polymorphic because it is able to exist in yeast, hyphal, or pseudohyphal forms and is the primary species responsible for candidiasis of the oral mucosa, skin, and genitalia. Despite being a human commensal, *C. albicans* and other non-*albicans Candida* spp. are able to cause severe disease in immunosuppressed patients (31–34). Filamentous growth is required for host tissue adhesion and penetration. Although by definition, pseudohyphae are considered to be elongated blastospores, their contribution to tissue damage and penetration along with true hyphae has also been confirmed by numerous studies.

Commonly occurring *Candida* spp. that are able to exist in all three forms, in addition to *C. albicans*, include *Candida dubliniensis* and possibly *Candida tropicalis*, while species capable of switching between only yeast and pseudohypae are *Candida parapsilosis*, *Candida guilliermondii*, and *Candida lusitaniae* (35).

Other examples of ascomycetes reported to be dimorphic and frequently associated with pathogenic behavior are *Exophiala dermatitidis* (Chaetothyriomycetes), *Lacazia loboi* (Eurotiomycetes), and *Aureobasidium pullulans* (Dothideomycetes). All of these species are associated with subcutaneous infections. Besides ascomycetes, morphotype-associated pathogenicity is also observed among basidiomycetes that infect mammalian hosts. Although *Cryptococcus neoformans* is not considered to be a dimorphic fungus, this species has a unique property of forming "giant," or "titan," cells in the host. Further studies have also described morphotype-associated pathogenicity among certain Zygomycota and dermatophyte species (36, 37).

Plant Pathogenic Dimorphic Fungi

Dimorphic fungi are also common pathogens of important crops. In contrast to pathogenic fungi of mammals, phytopathogens are usually considered saprobic in the unicellular yeast phase but parasitic in the filamentous stage inside the plant. The role of the yeast form in pathogenicity is species specific and may contribute to dissemination (38). In the majority of cases, to penetrate the structural barrier of plant cells, hyphal growth is needed. Examples include *Taphrina deformans*, a common phytopathogen responsible for peach leaf curl, and *Ustilago maydis*, which is associated with corn smut disease. Once reaching the plant surface, both *U. maydis* and *T. deformans* switch from budding yeast to filamentous growth (21). The phenomenon is induced by mating in *U. maydis*, although not in *T. deformans* (39, 40). *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* are the main causative species of Dutch elm disease. Additional factors that promote morphology change in *Ophiostoma* spp. include lipoxygenases, cyclooxygenases, nitrogen sources, and quorum sensing molecules (41). Filamentous forms of *Ophiostoma* spp.

invade between the vascular structures, while blastospores disseminate in the xylem network of the infected elm trees.

Current scientific research is concerned about exploring the underlying conditions and exact altered signaling mechanisms that help dimorphic fungi to adapt to the host environment. Several mechanisms for yeast-hyphal switches have already been revealed, although our full understanding of morphotype-associated pathogenicity and thus the origin of virulence is still incomplete.

FRUITING BODIES

Fruiting bodies are some of the most spectacular morphological structures of fungi. Compared to indeterminately growing vegetative mycelia, sexual fruiting bodies result from temporally and spatially integrated developmental programs. In this regard, they resemble multicellular animals and plants, but their role in the species' life cycle is different. Whereas for plants and animals the complex multicellular phase of the life cycle comprises the reproducing individual, the role of fungal fruiting bodies is restricted to sexual reproduction. Therefore, unlike vegetative mycelia, fruiting bodies are quite ephemeral; their life span usually ranges from a few hours to a few days (except highly resistant bracket fungi, which can be perennial). They support the development and dispersal of meiospores in terrestrial environments by providing protection and a skeleton on which spore-producing basidia or asci develop.

Fruiting bodies of some sort evolved in most clades of terrestrial fungi (Fig. 1A,C), but it is the Dikarya in which the most typical manifestations and >99% of the fruiting body-forming species are found, with the two largest groups being the Agaricomycetes and the Pezizomycotina. It is less well known that fruiting bodies have evolved in several other clades as well, such as the Taphrinomycotina, the Pucciniomycotina, and the "Zygomycota." The earliest diverging clades in which fruiting bodies developed are the Mucoromycotina and the Mortierellomycotina, to which *Endogone* spp. and *Modicella* spp. belong, respectively. These species form small irregularly lobed or truffle-like structures that contain zygospores (42). Fruiting body-forming members of the Pucciniomycotina (rusts and allies) usually form up to a few millimeters-tall cylindrical or capitate structures (e.g., Phleogena, Gymnosporangium) or crust-like, corticioid layers on the substrate (e.g., Septobasidium). The Taphrinomycotina contain the genus *Neolecta*, one of the most enigmatic fungi, which forms vividly colored, tongue-like ascocarps (6, 43).

Although fruiting bodies can be found in most clades in the Dikarya and even in earlier-diverging clades, the evolutionary origins of their development are not well known. The patchy phylogenetic distribution of fruiting body-forming fungi suggests multiple independent origins, which is further supported by the apparent lack of homologies between fruiting bodies in separate clades. Given the widespread occurrence of fruiting body-producing lineages, however, the alternative hypothesis that the last common ancestor of all Dikarya species was able to produce fruiting bodies cannot be ruled out.

Diversity and Evolution of Fruiting Body Types

Fruiting bodies serve two major goals: the protection of developing reproductive organs and the promotion of spore dispersal either by providing an enlarged supporting surface for the sporogenous tissue (e.g., hymenium) and/or by lifting it above ground level. Several alternative solutions have evolved for these tasks during evolution, ranging from simple aggregations of basidia or asci on a hyphal mat (subiculum) to highly integrated complex types that consist of several (>10) distinct cell types (44). The simplest such solutions in the Basidiomycota comprise crust-like, flat fruiting bodies that enclose basidia into a sporogeneous tissue (resupinate type [45–47], e.g., *Phanerochaete*, *Peniophora*), whereas in the Ascomycota, cleisthothecia (closed sac-like structures) are the simplest fruiting body types (43, 48). Relative to these simple types, a graded series of increasingly more complex morphologies have evolved in several clades (Fig. 1C-E), which will be detailed in the next paragraphs.

The evolution of fruiting body types has followed different principles in the Asco- and Basidiomycota. In the Basidiomycota a clear trend exists from crust-like, resupinate, to more complex morphologies (45–47). A great diversity of morphological types derived from the ancestral resupinate types, including coral-like (coralloid), pileate-stipitate (those with cap and stipe), console-shaped (polyporoid and bracket fungi), puffball-like (gasteroid), or reduced cup-shaped (cyphelloid), among others. The pileate-stipitate, typical "mushroom-like" morphologies evolved several times from resupinate ancestors (45–47, 49), possibly through coral-like intermediates (50). The pileate-stipitate morphology appears to be a (sub)terminal state, dominating the largest Basidiomycete orders (Agaricales, Boletales, Russulales), possibly due to an optimized surface enlargement of the spore-producing tissue and to efficient protection from precipitation. Pileate-stipitate fruiting bodies show

few evolutionary transformations, with the exception of gastromycetation and sequestrization, two related processes denoting the origins of puffball-like fruiting bodies from agaricoid ancestors (46, 49, 51). These evolve by the gradual sequestration of the hymenium and often evolve toward an underground, truffle-like lifestyle, probably as an adaptation to dry environments and to zoochory (dispersal by animals). Gasteroid forms in the broad sense (including secotioid and sequestrate) evolved several times from pileate-stipitate ancestors, although they rarely dominate their clades in terms of species diversity, suggesting an internal genetic tendency for their evolution that is coupled with mechanisms that limit their diversification. Another widespread type derived from pileate-stipitate ancestors is the cyphelloid morphology (52). These are cup- or tube-shaped pendant fruiting bodies that most likely evolved via the loss of the stipe and gills of agaricoid species. They are found in several, mostly white-spored clades of the Agaricales.

Ascomycete fruiting bodies evolve from open morphologies toward closed fruiting body types (53). The major morphological types found in the Ascomycota are cleistothecium, perithecium, apothecium, and pseudothecium. The simplest of these is the cleistothecium, a completely closed, usually globose structure that often bears characteristic hyphal appendages. Cleistothecia are characteristic of the Eurotiomycetes (*Eurotium*, *Talaromyces*, and allies). Perithecia are globose or flask-shaped structures that open to the outside through the ostiolum, a channel through which spores exit the fruiting body. Perithecia are rarely bigger than a millimeter but can develop in large numbers and can congregate on a common supporting stroma (e.g., in *Xylaria* species). They are characteristic of the Sordariomycetes and the Laboulbeniomycetes (derived perithecia). Pseudothecia are morphologically similar to perithecia, but they often incorporate tissues from the substrate, and asci are not regularly organized within them. They are found in the Dothideomycetes. Apothecia are completely open, plesiomorphically cup- or disc-shaped fruiting bodies that can reach several centimeters in size. They are found in species of the Pezizomycetes, Leotiomycetes, Orbiliomycetes, Lecanoromycetes, and Lichinomycetes. Several modified and highly derived complex versions of apothecia have evolved, such as morels (*Morchella* spp.) and truffles (e.g., *Tuber* spp.). Like gasteroid fruiting bodies of basidiomycetes, truffle-like species evolved several times from apothecial ancestors, probably in response to some widespread selection pressure such as dry climate (54).

Development of Fungal Fruiting Bodies

The development of fruiting bodies on vegetative mycelia is a major transition in the life of a fungus. It involves a complete reprogramming of hyphal branching patterns and the initiation of a spatially and temporally organized developmental program. Initiation of fruiting body development is part of the sexual reproductive cycle and is thus regulated by genes located in the mating type loci and involves the recognition of compatible nuclei, which happens in basidiomycetes when dikaryotic hyphae develop (48, 55).

Both ascomycete and basidiomycete fruiting bodies develop from aggregated hyphae. In the basidiomycete *Coprinopsis cinerea*, which has been used extensively to study fruiting body development, the first hyphal aggregates are primary hyphal knots, which differentiate primarily in response to light and nutritional signals. Secondary hyphal knots derive from primary hyphal knots, and their differentiation is controlled by both environmental factors and the activity of mating type genes (55, 56). Secondary hyphal knots develop into stage 1 primordia (0.5 to 1 mm diameter), which already have cap, stipe, and gill rudiments and develop further into stage 2 primordia (2 to 3 mm tall), then to young and mature fruiting bodies. A characteristic of *Coprinopsis* spp. is an autolytic phase at the end of development, during which endogenously produced chitinase and glucanase enzymes digest the tissues into a black inky liquid, which might facilitate spore dispersal but may also have other roles (57). In the ascomycete *N. crassa*, the development of fruiting bodies starts from protoperithecia. Female protoperithecia are induced by nitrogen starvation, fertilized by a compatible male cell (hyphal fragments, micro- or macroconidia), and develop into mature perithecia that contain asci and eight ascospores (48). Variations of these developmental scenarios in various species exist, but the general principles of the early development seem to be conserved across the Asco- and Basidiomycota.

In pileate-stipitate species of the Basidiomycota, a number of developmental types have evolved, differing in the spatial and temporal patterns of tissue differentiation and the extent to which primordia are covered by protective tissues (veils and blemas), among other factors. Based on where in the fruiting body initial primordial cap tissues emerge, epi- and endonodular development can be distinguished (58). In epinodular species, cap rudiments develop on top of the nodulus (globose structures derived from hyphal knots), whereas in endonodular species, the cap is formed within the nodular context. Epinodular development is further divided into exocarpic, where cap and hymenium rudi-

ments are open to the environment, and endocarpic, where the developing hymenial tissues are covered by various hyphal layers. In traditional terms, endonodular and endocarpic types correspond to angiocarpic, whereas exocarpic corresponds to gymnocarpic development, although a strict correspondence is difficult to draw. These protective hyphal sheaths can later vanish or give rise to universal and partial veils, leaving the characteristic patches and rings on mature mushrooms, respectively (58).

Fruiting body morphologies and developmental patterns formed the basis for mushroom classification for a long time. For example, gasteroid basidiomycetes have been classified in the Gasteromycetes on the basis of their shared gross morphology and closed development, although several anatomical, biochemical, or ultrastructural characteristics pointed at a potentially polyphyletic origin (59, 60). Fungal classification has been revolutionized by molecular phylogenetic studies, yet developmental characteristics are still useful for species identification and for defining monophyletic groups.

The Genetic Bases of Fruiting Body Development

Fruiting body development is realized by intricate networks of regulatory and structural genes. A large number of signal transduction pathways and gene regulatory networks orchestrate responses to external (e.g., nutrient availability, light, temperature) and internal (e.g., pheromones) cues (for reviews see references 48, 56) that ultimately lead to the reorganization of the expression patterns of structural and effector genes. Among the environmental stimuli, light is central to the development of fruiting bodies. Light responses are orchestrated to a large extent by blue light receptors, primarily by the photosensor white-collar genes (WC1-2) first identified in *N. crassa* (56, 61, 62). One of the key regulatory pathways of fruiting body development is the velvet complex (VelB, VeA, and LaeA in *A. nidulans*), which controls secondary metabolism and sexual reproduction in response to light stimuli (63, 64). The striatin-interacting phosphatases and kinases complex has recently been characterized in *Sordaria macrospora* as a significant component of internal signaling pathways involved in fruiting body development (65, 66). Several transcription factors impact fruiting body development, morphology, and morphogenesis (for more details see references 67–70). Structural and effector genes are recruited from the cellular morphogenesis toolkit, among other genes for cell wall modifications, cytoskeletal proteins, and genes involved in protein and cell wall component biosynthesis or coding for cell wall-associated proteins. A number of fruiting body-specific proteins have also been identified, in particular, cell wall proteins such as lectins (71, 72), various lectin domain-containing proteins, and hydrophobins (73). The roles of several developmentally regulated, but as of now poorly characterized, genes await more thorough understanding. For example, the PriA and Fve proteins were found to be overexpressed in primordia and young fruiting bodies of *Lentinula edodes* and fruiting bodies of *Flammulina velutipes*, but their exact role in the developmental process remains an enigma.

PLANT CELL WALL DECOMPOSITION BY FUNGI

Most fungi obtain their nutrients from plants, either as saprobes, parasites, or mycorrhizal mutualists. They evolved a wide array of extracellular enzyme systems to attack and decompose complex plant materials and are the most efficient decomposers of lignocellulosic plant cell walls among all microbes. Thus, fungi play an important role in reintroducing fixed carbon into the carbon cycle by degrading dead plant debris and contribute to the functioning of ecosystems all over the world. Several symbiotic interactions also rely on the ability of fungi to decompose plant cell walls. For example, fungus-growing termites and ants use the lignocellulose-degrading ability of various *Leucoagaricus*, *Termitomyces*, and *Pterula* spp. (Agaricales), whereas ruminants harbor large populations of anaerobic Neocallimastigomycota fungi in their rumen. Fungal lignocellulose decomposition is achieved by a variety of mechanisms, including both enzymatic and nonenzymatic systems. The extracellular enzymes of wood-decay fungi received intense industrial interest because of their potential to facilitate the bioconversion of lignocellulosic materials for biofuel production and other industrial applications (e.g., paper industry).

The Diversity of Wood-Decay Types

Wood decay strategies have been classified in three broad categories in the past: white rot, brown rot, and soft rot (Fig. 1A,C). White rot fungi degrade cellulose, hemicellulose, and lignin components of plant cell walls (74), whereas in soft and brown rot only cellulosic substances are removed and lignin is not appreciably degraded. White and brown rot are typical of Basidiomycetes, whereas soft rot is mainly produced by ascomycetes. This broad classification of decay types has been contested recently based partially on comparative

genomics of Basidiomycota (74–76), suggesting that the functional diversity of wood-decay fungi and their role in ecosystems could be better described by a continuum. Several species traditionally classified as white rot fungi lack the enzyme systems to degrade lignin. Among others, *Schizophyllum commune*, *Botryobasidium* spp., and *Jaapia* spp. belong to this category, and although they are usually found on white-rotted wood, their nutritional strategy is unclear. Because of this uncertainty in decay mode and the diversity of nutritional strategies, the definition of white rot is now restricted to species that achieve lignin degradation through the action of class II peroxidases (76) (see below). In general, it is likely that a wide array of strategies for attacking lignocellulose-containing plant cell walls have evolved in various fungi and that these capabilities will support diverse industrial applications when it is known how to leverage them.

The capacity for white rot presumably evolved in the most recent common ancestor of the Auriculariales and more-derived Agaricomycetes (74, 75). Species associated with white rot (e.g., *Botryobasidium* spp., *Tulasnella* spp.) can be found in earlier-diverging clades too (Cantharellales), but they lack the hallmark enzymes of white rot (76). The symptoms they produce on rotten wood might reflect an ancestral, soft-rot-like wood decomposition strategy (75). White rot has a single evolutionary origin but has been lost several times. Brown rot fungi, which evolved by the loss of much of the white-rot-specific enzyme complements, originated several times in the Agaricomycetes, among others in the Polyporales, Hymenochaetales, Boletales, and Agaricales (74). Their convergent evolution is underpinned by the loss of several lignocellulose-degrading enzymes, in some cases the loss of complete enzyme/protein families.

The Evolution of Decay-Related Enzyme Families

Enzyme families involved in lignin decomposition are overrepresented in the genomes of white rot fungi (74–79) compared to brown rot and mutualistic fungi. Phylogenetic analyses of these gene families revealed an intricate evolutionary history formed by gene duplications and losses throughout the Basidiomycetes. Numerous gene duplications have been reconstructed in lineages containing white rot fungi, generating the raw material for the evolutionary diversification and potentially for adaptations to various substrates and environmental conditions. However, whether the enormous diversity of these enzymes within the same genome (e.g., up to 40 copies of peroxidases in a genome) reflects a diversity in the expression, activity, or substrate specificity across multiple similar copies is not well understood.

The ability to decay wood components was probably already present in the most recent common ancestor of the Dikarya (Ascomycota plus Basidiomycota), some 700 to 750 million years ago (74, 80–82), and maybe even earlier. However, it was not until the end of the Carboniferous when high redox-potential enzymes and thus white rot fungi evolved and started to degrade woody substrates on Earth (74). Although molecular clock dating of fungal divergences is notoriously difficult, recent genome-based analyses support the origin of the Agaricomycetes around 290 million years ago. The diversification of white rot fungi at the end of the Carboniferous has been linked to the decline in organic carbon accumulation in this era (74, 83).

In contrast to white rot fungi, brown rotters degrade the cellulosic materials through nonenzymatic action involving H_2O_2 produced by the Fenton reaction. Brown rot fungi possess very few or no ligninolytic enzymes due to extensive gene losses in these gene families, which might be driven by selection for the loss of energetically expensive ligninolytic mechanisms of white rot species (84). Although not able to utilize lignin for energy production, brown rot fungi evolved mechanisms to modify lignin so that cellulose fibrils among lignin polymers become more accessible (85). Brown rot fungi are also adapted better to coniferous tree hosts, in contrast to white rot fungi, which are more often found on angiosperm wood (86, 87).

Beyond lignocellulose-active enzymes, other cellular pathways are at least as important for the utilization of plant cell wall materials, such as detoxification and membrane transport pathways for neutralizing toxic decomposition intermediates and importing monomers into the cells. These pathways, however, received less attention than the front-end enzyme systems and will require greater scrutiny in future studies.

The Cellular and Genetic Bases of Wood Decay

Extracellular enzymes play a major role in the degradation of lignocellulosic compounds of plant cell walls by wood-rotting fungi. Plant cell walls are complex and highly diverse composites of various polysaccharides. Fungi evolved a similarly diverse suite of enzymes, mostly glycoside hydrolyses for degrading these components, the most important of which are cellulases, hemicellulases, pectinases that act on main chains of polysaccharides, and accessory "debranching enzymes" that digest polysaccharide side chains. Ligninolytic en-

zymes (87–89) decompose highly recalcitrant lignin polymers. Enzyme families acting on cellulose chains include endoglucanases, exoglucanases, and beta-glucanases. The oxidoreductive cleavage of cellulose chains by lytic polysaccharide monooxigenases has only recently been discovered as an additional synergistic mechanism of cellulose degradation (90, 91). The decomposition of hemicellulose and pectin, due to their complex structure, involves a more diverse set of enzymes: xylanases, mannanases, galactosidases, endoglucanases, cellobiohydrolases, beta-glycosidases, endo- and exopolygalacturonases, pectin and pectate lyases, etc (92). Cellulose, hemicellulose, and pectin degradation have evolved in both the Asco- and Basidiomycota, but there are large differences in the individual species' ability to decompose these compounds. Pectinases, for example, were probably present in the genomes of ca. 750-million-year-old aquatic ancestors of the Dikarya and Chytridiomycota, indicating an ancient adaptation to obtain nutrients from pectin-containing cell walls of early green plants (82).

Lignin, one of the most recalcitrant biopolymers found in nature, is composed of cross-linked aromatic phenol-polymers. The most efficient decomposers of lignin are white rot fungi belonging to the Agaricomycetes, wherein members of the order Polyporales contain the best-characterized white rot species (e.g., *Trametes versicolor*, *Phanerochaete chrysosporium*). Less well known but important lignin decomposers are also found in the Agaricales, Boletales, and Russulales. Lignin degradation by white rot fungi is achieved through the generation of oxidative radicals by extracellular enzyme systems. The most important ligninolytic enzymes are class II peroxidases, laccases, and dye decolorizing peroxidases. Based on structural and functional considerations, class II peroxidases are further subdivided into four subfamilies: lignin peroxidases, versatile peroxidases, manganese-peroxidases, and generic peroxidases (74, 89, 93). These high-redox-potential peroxidases catalyze the oxidation of lignin polymers by hydrogen-peroxide generated by glyoxal oxidases, pyranose oxidases, and aryl-alcohol oxidases (93, 94).

SECONDARY METABOLISM

Secondary (or specialized) metabolites (SMs) are small molecules whose primary roles involve ecological or environmental interactions as opposed to basic growth, development, and reproduction. For example, SMs secreted by fungi may act as antibiotics or signals to other organisms or alter the availability of nutrients in soil or host tissues. Fungal SMs can be quite diverse, similar to plants, and are in part distributed among fungi according to ecological traits rather than species relationships. The origin and evolution of secondary metabolic pathways in fungi reflect broader patterns of genome remodeling in a biochemical arms race with competitors and hosts.

Only a small proportion of the adaptive biological functions of SMs are characterized (it is sometimes argued that most have no current functions [95]), but a subset of these is known to have important ecological roles (96). As with plants, the most obvious roles for SMs are defense and competition (Fig. 2). Although most classes of antibiotic pharmaceuticals are bacterial in origin, antibiotics are nonetheless important products of fungi (97). Beta-lactams, including penicillin and cephalosporins, are among the few fungus-derived antibiotics that are suitable for human use. The existence of a large number of SMs is suggested by homologs of known SM genes found in the growing database of whole-genome sequences, but they have not yet been observed to be expressed (98). Cryptic antifungal and antibiotic fungal SMs can be uncovered via dual-plating experiments, in which fungi paired with other microorganisms secrete compounds that inhibit growth of the competitor or via inducing the expression of "silent" SM genes (99). However, specific SM-mediated ecological interactions are not usually documented in their natural context.

Among the main exceptions to this lack of tested functionality are SMs produced by fungi that act as pathogens of plants. For example, plant pathogens often secrete SM toxins to directly induce plant cell death during necrotrophic growth (100). *Cochliobolus carbonum* secretes the host-selective HC toxin, which prevents induction of normal defenses in maize (101). Other plant pathogens manipulate the host's internal signals involved in growth and programmed cell death. For example, *Botrytis cinerea* and other fungi secrete the plant hormones abscisic acid and ethylene during necrotrophic pathogenesis (102).

Some fungal SMs appear to be involved in fungus-animal interactions. Nematodes and arthropods are the main predators of fungi, so it is likely that they are often the targets of SMs that affect animal neurotransmitter signaling pathways. For example, psilocin and muscarine are produced by a number of mushroom-forming basidiomycetes and bind to serotonin and acetylcholine receptors, respectively (103). Ascomycete molds also produce larvicidal compounds, and a mutant *Aspergillus* that produces few SMs due to deletion of a global regulator of SM pathways is preferred by fungivorous arthropods (104).

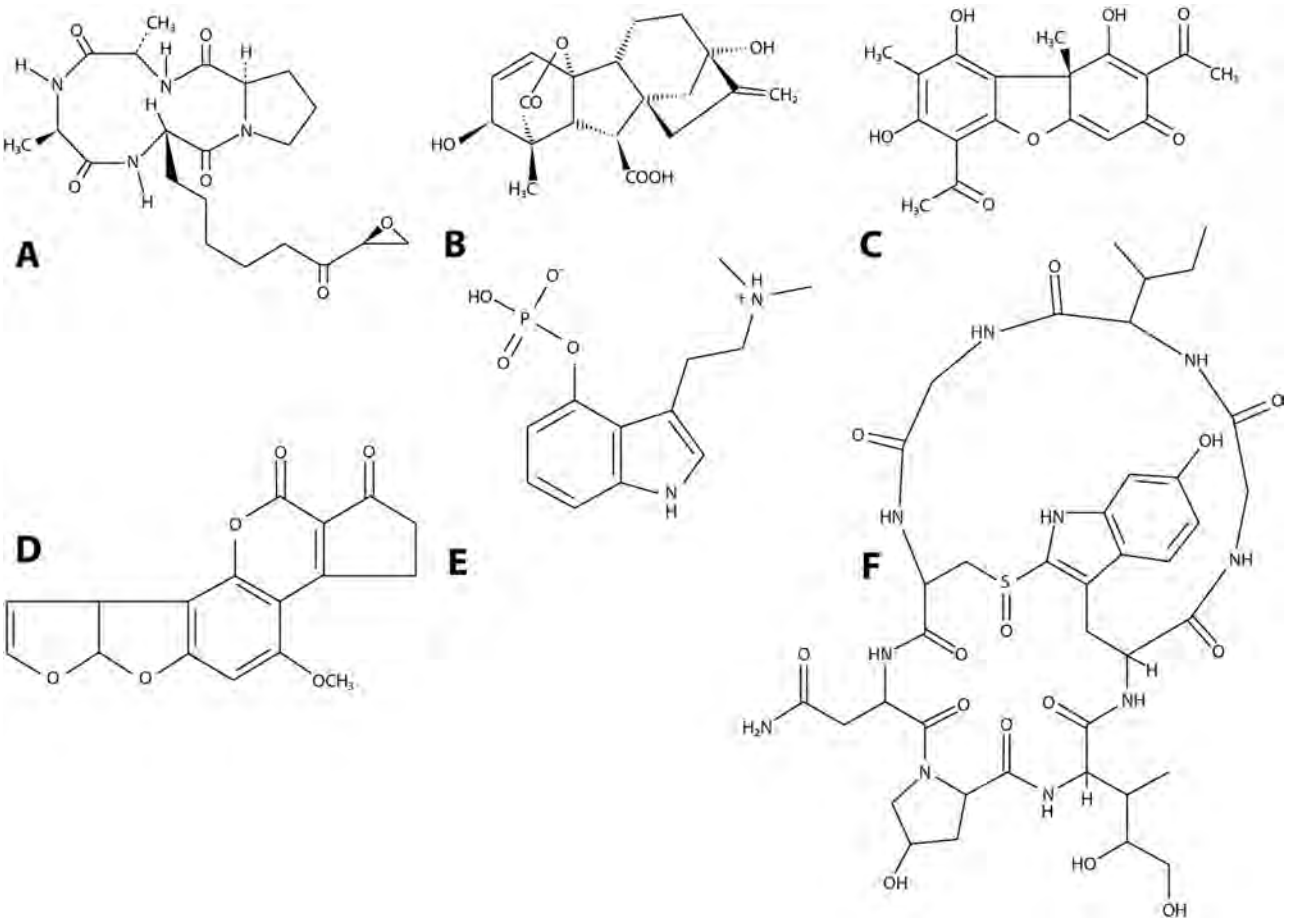


Figure 2 Secondary metabolites produced by fungi. (**A**) HC toxin is a nonribosomal peptide virulence factor produced by plant pathogens such as the Northern corn leaf spot fungus, *Cochliobolus carbonum*. (**B**) Gibberellic acid is a diterpene-derived plant growth hormone produced by some *Fusarium* spp. (e.g., *Fusarium moniliforme* = *Gibberella fujikuroi*), which leads to growth defects in grass seedlings. (**C**) Usnic acid is a common polyketide secondary metabolite thought to protect against biotic and abiotic stress in many lichens. (**D**) Aflatoxin B1 is a highly carcinogenic polyketide produced by *Aspergillus* molds that spoils grain and peanut harvests. (**E**) Psilocybin is a hallucinogenic indole alkaloid produced by a variety of mushrooms. (**F**) Alpha amanitin is a deadly peptide alkaloid that inhibits RNA polymerase II, produced by diverse mushroom-forming fungi.

These same antimicrobial and competitive functions can also be of benefit to plant hosts of symbiotic fungi. Grass endophytes of Clavicipitaceae have long been studied for their antiherbivory toxins, which can increase fitness and dominance of infected plants (105). Surprisingly, some endophytes have been recently shown to produce the same SMs as their hosts and may compensate for reduced production of these SMs by the plants when they are allocating resources to, for example, reproduction (106). In general, endophytes are prolific producers of diverse metabolites and are considered "acquired immune systems" of the plants that benefit from them (107).

Because fungi are absorptive-feeding organisms, fungal growth is constrained by environmental conditions and competition that affect the availability of nutrients. SMs play a role in increasing access to limiting elements such as iron. Siderophores are molecules with especially high affinity for binding iron (108). There are a number of independently evolved siderophores in fungi. Siderophores are particularly important to animal-pathogenic fungi that compete with both hosts and their commensal microorganisms for limited iron (109, 110).

SMs are also important for protection from environmental stresses such as desiccation and radiation.

Melanins are amorphous SM polymers synthesized from various precursors, which absorb radiation and create a resistant barrier to dehydration and mechanical damage (111). For example, the melanized rhizomorphs of *Armillaria* spp. allow the fungus to cross long distances in exposed, low-moisture environments to access new substrates (112), and melanized sclerotia enable pathogens and symbionts of annual plants to survive and overwinter in detritus and soil. The tendency of melanins to neutralize radical molecules may also protect melanized yeasts against similar molecules used by immune macrophages and soil predators such as amoeba (113). Lichenized fungi, on the other hand, endure chronic environmental exposure and consequently utilize numerous SMs to reduce the damaging effect of light exposure (114).

Major Types of Secondary Metabolites

Five main classes of fungal SMs have been studied in depth: peptides, polyketides, peptide-polyketide hybrids, terpenes, and alkaloids. Peptide SMs are usually synthesized by special polymerizing enzymes called nonribosomal peptide synthetases but are occasionally produced from repetitive DNA templates by cleavage of the resulting polypeptide sequences. Examples of nonribosomal peptide synthetase-produced SMs include the beta-lactam antibiotics such as penicillin and cephalosporin and the immunosuppressant cyclosporin (115). Amatoxins, the deadly RNA transcription inhibitors produced by death angel mushrooms such as *Amanita bisporigera*, are produced by cleaving 35 amino acid proteins into octopeptides, which are then cyclized by a mechanism that is not fully understood (116). Polyketide synthetases, the most abundant SM-producing enzymes in fungi, polymerize fatty acid subunits rather than amino acid subunits like the nonribosomal peptide synthetases (115). A basic polyketide chain can be modified with functional group "decorations" and formed in rings by other enzymes in these pathways to produce a wide range of compounds. Among these are the aflatoxins from *Aspergillus* spp., which are toxic and costly contaminants of maize, peanut, and other crops (117), and statins from diverse species, originally targeted as pharmaceuticals to reduce cholesterol synthesis (118).

Terpenes are volatile or nonvolatile compounds produced from isoprenoid subunits and include both the trichothecene mycotoxins from *Fusarium* spp. and desirable flavor compounds in edible mushrooms (119, 120). Gibberellic acids are terpenoid hormones first characterized in *Gibberella* (*Fusarium*) *fujikuroi*-infected rice as the cause of hypertrophy symptoms leading to extensive crop losses. Alkaloids are diverse nitrogen-containing SMs that are often associated with toxicity and feeding deterrence against herbivores and fungivores. Vertically transmitted fungal endophytes of grasses, for example, produce from amino acid precursors four classes of alkaloids that are toxic to mammals and invertebrates. Among these, insecticidal loline alkaloids are derived from L-proline and L-homoserine, and livestock/human-intoxicating indole and indole diterpene alkaloids, which interfere with neural and vascular processes, are derived from L-tryptophan (121). Notable toxicoses caused by indole-based alkaloids include "ryegrass staggers" in livestock caused by lolitrem B, and ergotism and related syndromes in humans and animals caused by ergot alkaloids produced by *Claviceps purpurea* (ergot) and clavicipitaceous grass endophytes. Psilocin from "magic" mushrooms and lysergic acid diethylamide (LSD) are indole alkaloids that have additionally influenced human culture and religion through their hallucinogenic activity (122). Due to their high potential for bioactivity, fungal alkaloids have contributed a number of therapeutic drugs such as ergometrine, an ergot alkaloid that revolutionized medical control over postpartum hemorrhage, and a number of promising anticancer drugs.

The Evolution of Secondary Metabolism in Fungi

The roles and extent of secondary metabolism in fungi vary widely by lineage. Filamentous Ascomycota contain by far the majority of known SM genes, while ascomycete yeasts, Basidiomycetes, Zygomycetes, and chytrids have few or none of the classes described above (123). Fungal metabolic pathways involved in ecological processes are often encoded in "gene clusters" (124). For example, most enzymes, regulators, and transporters required for production and delivery of SMs are the products of genes in close physical proximity on chromosomes. The evolution of such gene clusters is highly dynamic; specific gene clusters are usually distributed among only a fraction of fungal taxa but can be found in taxa that are distantly related (125). This distribution of gene clusters reflects a history of horizontal gene transfer, along with rapid degeneration, loss, and remodeling of metabolic pathways. Numerous SM gene clusters appear to have been horizontally transferred between distantly related fungi (126, 127). For example, the 24-gene sterigmatocystin cluster in *Podospora anserina* (Sordariomycetes) is highly similar to that in *A. nidulans* (Eurotiomycetes) both in gene order and nucleotide sequence (128). However, within *Aspergillus*, homologous clusters are variable in their

presence and composition. Rapid rearrangement and degeneration of these clusters resulted in a diversity of producers and nonproducers of aflatoxins among *Aspergillus* species and the virulence factor dothistromin in the pine pathogen *Dothistroma septosporum* (129). Secondary metabolism gene clusters in fungi will continue to be an important topic of evolutionary, ecological, and pharmacological studies in the coming years.

MYCORRHIZAE

One of the major traits of fungi is their ability to form symbiotic interactions with plants. These interactions can be either parasitic or mutually beneficial, and many of them probably evolved in parallel with the colonization of the land by plants. It has been long suggested that the colonization of land by plants has been aided by early mutualistic fungi (130). There are several types of mutualistic interactions between plants and fungi, among which mycorrhizae formed by land plants and fungi are the most frequent. Although plant-fungal symbioses played a crucial role and were a driving force in the evolution of both partners, here we focus on the evolution, physiology, and anatomy of mycorrhizae from a fungal perspective.

There are several possible definitions of "mycorrhizae," and while there might be debates about them, the main types of mycorrhizal interactions are relatively well defined. The term itself means "fungus-root" and was coined by Frank (1885) in his landmark work in which he also posited the importance and mutual benefits of reciprocal nutrient exchange through mycorrhizae (131, 132). According to Trappe's definition (179), mycorrhizae are "dual organs of absorption formed when symbiotic fungi inhabit healthy absorbing organs (roots, rhizomes, or thalli) of most terrestrial plants and many aquatics and epiphytes." The use of the phrase "most terrestrial plants" is not exaggeration at all: the majority of land plants form mycorrhizae: 90% of the plant families are mycorrhizal, as are 70 to 80% of known plants on the species level (133–137). The main function of mycorrhizae is reciprocal nutrient exchange between the partners: using their extraradical hyphae, fungi mobilize and/or uptake nutrients from the soil, transport them into the roots, and thus to the plants, while the plants transfer assimilated sugars to the fungal partner (133, 138). The most important nutrients mobilized and transported from the soil are phosphate, nitrogen, and water. Nutrient mobilization, however, depends on the type of mycorrhiza, the fungal partner, the habitat, and the environment. Hence, there is a fine balance in the dominance and importance of different processes, mycorrhizal partners, and the main nutrients exchanged between them in different habitats and ecosystems (133, 138, 139).

Diversity of Mycorrhizal Types

Coining a generic definition of mycorrhizae is complicated by the functional and anatomical diversity of mycorrhizal interactions. The wide range of morphological types of mycorrhizae can be classified as two main forms of fungal colonization: in endomycorrhizae the fungal hyphae penetrate the root cells, whereas in ectomycorrhizae (ECM) the fungi colonize the intercellular spaces only. There are clear differences between these two types, not only in gross anatomy, but also in characteristic enzyme sets used by the fungi. Endomycorrhizae can be further subdivided into arbuscular mycorrhizae (AM), orchid mycorrhizae, and ericoid mycorrhizae (ERM), each characteristic of certain plant lineages (133, 134). Many other types characteristic of smaller plant groups exist (134). For example, arbutoid and monotropid mycorrhizae are ectendomycorrhizae showing main structural characteristics of ECM but also hyphal colonization of plant cells (133). The plants forming these mycorrhizae are mycotrophic: they are not or are not completely autotrophic and gain assimilates from other plants mostly through connections with ECM fungi.

ERM are endomycorrhizae formed by plants in the Ericales and various asco- and basidiomycetes (133, 134). The best-known fungal taxa are found in the ascomycete genera *Hymenoscyphus*, *Cadophora*, and *Oidiodendron*, but *Sebacina* spp. and other basidiomycetes have also been identified as ERM fungi (140). Some Ericales have specialized, extremely thin, simple roots (133, 141) and grow in habitats with a very slow organic turnover, which makes them dependent on mycorrhizal partners with good saprobic capacities.

Orchid mycorrhiza (ORM) is also an endomycorrhizal interaction, with characteristic intracellular hyphal forms such as the pelotons (densely coiling intracellular hyphal mass) and the moniliform hyphae (133, 141). Orchids depend obligately on their symbiotic fungal partners. They require the presence of fungi for the germination of their seeds and the development of the protocorm stage (133). Some orchids are mycoheterotrophic due to the complete or incomplete loss of photosynthetic capacity in the adult stage or the insufficient amount of light in their habitats. These mycoheterotrophic species fully or partly rely nutritionally on fungi that form mycorrhizae with autotrophic plants (133). The decomposition processes driven by fungal partners with strong saprobic capacities also play a role

in the carbon supply to the plants in ORM. Although the association between orchids and fungi had been known for a long time, the mutualistic reciprocal transport was proven only 10 years ago: in addition to the fungal N transport to the plant, a reciprocal C exchange between the two partners has also been observed (142). The most widespread orchid mycorrhizal fungi are found in the Cantharellales (Basidiomycota), e.g., *Rhizoctonia*, *Tulasnella*, and *Ceratobasidium*. However, a huge diversity of other, mostly basidiomycete fungi can also be detected in orchid roots. Even members of the Atractelliomycetes (Pucciniomycotina) were proven to colonize and form an intracellular interface in terrestrial orchid roots (143).

Arbuscular Mycorrhiza (AM)

AM is the most widespread mycorrhiza type and probably the most frequent symbiosis on Earth (144, 145). AM is exclusively formed by obligate biotrophic fungi of the Glomeromycota, while high numbers of plant species from almost all mainland plant groups form this mycorrhizal type (133, 136). The Glomeromycota is a relatively small group with approximately 230 known species (146). One of the main benefits for the plants in this symbiosis is the phosphate transferred by AM fungi to the plants (133). Although these fungi harbor genes related to sexual reproduction (147, 148) no sexual stage is known. AM is an endomycorrhiza named after the tree-like arbuscules formed in the plant cells (133). Plant cells completely reorganize themselves during colonization by the AM hyphae, and the colonization itself depends highly on the plant, among other factors, because PCWDEs are missing from the genome of *Rhizophagus irregularis* (147). Precolonization recognition processes of plant and fungal partners in mycorrhizal interactions are best known in the case of the AM. Here, fungi release diffusible factors, e.g., lipochitooligosaccharides (the "myc-factors"), acting as elicitors and signals recognized by the plant, while the plants secrete diverse compounds such as strigolactons, which influence the fungus before colonization (149). The plant membrane around the arbuscules has many special characteristics; e.g., specific plant phosphate transporters are located on the periarbuscular membrane. The arbuscule and the perisymbiotic membrane share many similar features with haustoria of intracellular biotrophic pathogens such as downy mildews, powdery mildews, and rust fungi (150). Nevertheless, beneficial mycorrhizal and parasitic interactions have clearly different functional and regulatory features (150, 151).

Fossilized spores and arbuscular structures and the capability of the basal green plants to form AM (152–158) suggest that AM fungi helped plants to conquer land (159). The clade of AM fungi, Glomeromycotina (160), branched off approximately 400 to 450 million years ago in the phylogeny of fungi (161), and the Glomeromycotina groups with Mucoromycotina (160, 162, 163). This is especially interesting considering that Mucoromycotina fungi were recently shown to be mutualistic symbionts of basal plant groups (123, 164–166) and that hyphal structures resembling mucoralean fungi were also reported from plant fossils (167). These findings seem to change the paradigm that the Glomeromycotina was the only ancient mycorrhizal lineage. Nevertheless, it seems that the capacity to form AM evolved only once in fungal evolution, in the ancestor of the Glomeromycotina clade.

Although we have detailed data on the molecular mechanisms of AM symbiosis, ranging from the presymbiotic phase through root colonization and arbuscule development, and there is information about the shared genomic toolkits of AM-forming plants (168), our knowledge of the genomic backgrounds of AM formation is limited. For several reasons, including the relatively big (~140 to 150 Mb) genomes with a high number of repetitive elements, the genome of only a single species, *R. irregularis* (once known as *Glomus intraradices*), has been completely sequenced (147, 163).

Ectomycorrhiza (ECM)

ECM is the most dominant type in temperate forest ecosystems (133, 137, 169), although it can be abundant in tropical areas too (170). Interestingly, only around 2% of plant species, mostly woody plants (e.g., *Fagaceae*, *Pinaceae*), form ECM (133, 137). In ECM the fungal partner forms a mantle on the surface of the colonized root tips, and the exchange of nutrients takes place in an intercellular interface between the plant and fungus, called the Hartig-net. The Hartig-net is a coenocytic structure (171) formed around the epidermal and/or cortical cell layers of the roots (133). Based on the diversity in ECM morphology, including characteristics of emanating elements and rhizomorph structures, ECM have been assigned to different exploration types (172), which probably reflect functional differences among ECM fungi.

Several ECM fungal lineages appeared during the evolution of the Dikarya, to which the majority of ECM fungi belong. The capacity to form ECM evolved independently in the Basidiomycota (e.g., *Amanita muscaria*) and the Ascomycota (e.g., *Tuber melanosporum*), according to some estimates 78 to 82 times (137). Compared to AM, the ECM association is evolutionarily younger, around 100 to 170 million years old.

ECM groups have evolved from nutritionally diverse ancestors, including white rot and brown rot fungi and plant pathogens (173). The comparative analyses of the first two genome sequences of ECM fungi, the basidiomycete *Laccaria bicolor* and the ascomycete truffle *T. melanosporum*, revealed striking differences between the two ECM species (174, 175). This heterogeneity was further supported by the comparative analysis of 49 fungal genomes representing 11 ECM, 3 ORM, and 1 ERM species of fungi, alongside several saprobes and plant pathogens (173) and the genome of *Cenococcum geophilum*, one of the most widespread ECM-forming Ascomycota.

Consistent with the independent origins of ECM fungi, the genetic toolkits underlying the mycorrhizal interaction have also evolved independently. Thus, identifying the genetic bases of mycorrhiza formation is challenging, which is further complicated by the high proportion (7 to 38%) of orphan genes (i.e., genes showing no homology with any other known genes) induced during mycorrhiza formation. Their abundance might be explained either by the recent origins of the lifestyle and the underlying genes or by the high rate of the evolution of interaction-related genes. One conserved pattern across ECM lineages is the lower diversity of PCWDEs compared to related wood-decaying or parasitic species. Many lineages have lost most of their PCWDE complement, which indicates reduced saprotrophic capabilities of these fungi. Nevertheless, PCWDEs play a crucial role in root colonization and in nutrient mobilization in the soil, and their presence suggests that many lineages of ECM fungi retain some saprotrophic potential. In contrast to ECM, ERM and ORM fungi possess high numbers of PCWDE genes and have strong saprobic capacities (173). The genetic bases of mycorrhiza formation comprise complex pathways of secreted proteins, effectors, regulators, PCWDEs, and several hitherto unknown components. Small secreted proteins are particularly abundant in ECM species and are upregulated during mycorrhiza formation in high numbers (176). These might be involved in controlling host defense mechanisms during the establishment of the mycorrhizal interaction. For example, MiSSP7 of *L. bicolor* interacts with jasmonic acid signaling repressors of the host plant (this pathway modulates plant-microbe interactions in plants), which promotes symbiosis (177). The wide array of mycorrhiza-induced effector-like secreted small proteins suggests that several other aspects of regulating the interaction and maintaining the fine balance between mutualism and parasitism exist and remain to be identified (177, 178).

EVOLUTIONARY CONVERGENCE IN FUNGI

Fungi show evolutionary convergence at multiple levels. All of the traits considered here result from convergent evolution at various taxonomic levels. For example, fruiting bodies evolved in the Asco-, Basidio-, and Mucoromycota, but also show convergence at finer scales in the Basidiomycota, where the agaricoid morphology has evolved in at least seven orders independently. Convergence can be observed both in trait gains and losses, the latter being naturally more common and including the repeated evolution of brown rot lineages from white rot ancestors through losses of PCWDE genes or the parallel reduction of the multicellular phase in the life cycles of yeasts. On the other hand, surprisingly similar phenotypic solutions for polarized hyphal growth and fruiting body formation evolved several times independently. The evolution of ECM associations is marked by both gains and losses: many PCWDE genes have been lost, whereas genes involved in ECM formation have been gained in a lineage-specific manner in ECM clades (169, 173). It is therefore important to consider the genetic mechanisms of phenotypic convergence in fungi. Parallel losses of conserved genetic toolkits provide a straightforward explanation for the repeated evolution of similar phenotypes. For instance, the loss of ligninolyic and other PCWDE genes resulted in selective nonenzymatic degradation of cellulosic plant cell wall components by brown rot fungi, which presumably, combined with the parallel elaboration of as yet unknown compensatory biochemical activities, resulted in similar wood-degrading strategies in multiple clades of these fungi.

Similarly, the evolution of major yeast lineages has been marked primarily by gene loss and limited genetic novelty associated with the morphological transition. However, phylogenetic patterns suggest that phenotypic similarity evolved independently in multiple yeast-like clades, fruiting body-forming groups, or hyphae, which prefigures mechanisms for gaining similar phenotypes independently in a convergent or parallel manner. Consistent with the predictions of convergent evolutionary theory, independently evolved ECM lineages share little of the known genetic innovations involved in ECM formation (169, 173). On the other hand, classical convergence fails to explain the convergent evolution of polarized hyphal growth or fruiting body formation. For these cases, a continuum of mechanisms for convergent evolution needs to be considered, ranging from the parallel cooption of orthologous genes and pathways for similar functions to underlying latent homologies or the evolution of divergent genetic toolkits.

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What Defines the "Kingdom" Fungi?

3

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It is a certainty that the tree of life can be broken up into large units of biodiversity that span such great evolutionary distances that one could call them kingdoms. However, if these units are to have any meaning, they must represent radiations of diverse groups underpinned by unique shared derived adaptations that drove the evolutionary success of each group. Without such evolutionary characters, these classifications are merely abstract concepts or arbitrarily demarcated lineages. Even the five kingdoms (commonly called plants, animals, fungi, protists, and prokaryotes) that gained popular acceptance from Whittaker (1) were abstractly defined and nonmonophyletic. These kingdoms were defined based on cellular organization (prokaryote or eukaryote), multicellularity (protist or "higher" eukaryote), and trophic level (producer [plants], consumer [animals], or decomposer [fungi]). Somewhat more phylogenetically sound definitions (2) have replaced these classifications, but the names, defining characters, and number of kingdoms are still not settled (e.g., 3, 4).

When different authors classify higher units of biodiversity, there are few rules and many differing interpretations of what is important and what is not (see 1–3, 5–9); some authors have even abandoned the need for formal terms such as "kingdom" (10). Furthermore, mapping trait evolution onto the tree of life is difficult, and even more so when these determinations are subject to constant revision (i.e., characters mapped onto trees need to be repositioned because the tree is constantly being redrawn and the characters are found in additional taxa). The combination of these factors means that classifications such as kingdoms are temporary, and "noisy," especially with respect to placement of taxa that branch close to the border of a kingdom on a phylogenetic tree. Take, for example, the three primary monophyletic eukaryotic kingdoms: plants (Archaeplastida [10]), animals (Metazoa), and Fungi. It can be argued that, of the three, only the Archaeplastida have a known defining evolutionary trait that underpins their evolutionary success and diversification. This defining trait is the endosymbiotically acquired primary plastid organelle derived from a cyanobacterium (e.g., 11). However, even this trait is not unique, because the protist *Paulinella* (Rhizaria) also has a cyanobacterially derived endosymbiont with some characteristics of an organelle (12). For the Metazoa and Fungi, the distinction is much less clear between members of these kingdoms and their protist sisters, and historically, some authors did not separate fungi from protists (6). In the case of Metazoa, many of the cellular systems thought to define animal multicellularity have now been identified in their protist relatives (e.g., 13, 14), leading to a reevaluation of the cellular systems that define membership in the animal kingdom. In the following section, we discuss this conundrum with regard to defining the boundaries of the Fungi. We argue that, irrespective of where taxonomic boundaries are drawn or how we define the Fungi, understanding the changes that underpinned the evolution and diversification of this group is the most important and interesting aspect of this research area.

IN SEARCH OF A SYNAPOMORPHY FOR FUNGI

There have been numerous attempts to identify a genetic, molecular, or cellular character that defines the kingdom Fungi, specifically a synapomorphy that separates them from other groups. To formally qualify as a diagnostic synapomorphy, such a character must clearly define the clade (i.e., be held by the majority of the taxa that branch within the clade), in this case fungi. Secondary loss of a trait within the circum-

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scribed clade is formally permitted, but patterns of loss complicate the use of the trait as a synapomorphy. Importantly, the trait must be absent everywhere else in the tree of life. Historically, attempts to identify such characters have been met with limited success. Here, we summarize the several lines of evidence that have been put forward but ultimately refuted as characters used to define the Fungi.

Amino Acid Biosynthesis

For over 30 years, the synthesis of the amino acid lysine via the α-aminoadipate pathway was discussed as a putative diagnostic character for the Fungi, even though the presence of the pathway in *Euglena*, a photosynthetic protist, was also reported (15, 16). Access to eukaryotic and prokaryotic genomic data, and in some cases physiological experiments, has demonstrated that the genes encoding this pathway are present throughout the eukaryotes (17, 18) and, indeed, also present in a number of prokaryotic genomes (e.g., 19). These results indicate that synthesis of the amino acid lysine via the α-aminoadipate pathway is not a useful diagnostic character for the Fungi.

Ergosterol

One of the key genes for the α-aminoadipate lysine pathway encodes the enzyme α-aminoadipate reductase. This gene is involved in the synthesis of both lysine and ergosterol (18). The presence of ergosterol within the cell, and specifically the cell membrane, has also been claimed to be diagnostic for fungi (e.g., 20). Some authors have used this biomarker for tracing fungi from environmental samples (e.g., 21–23). Unfortunately, a large number of groups classified as Fungi, which are placed in phylogenetic trees within the fungal radiation, lack either the pathways to synthesize ergosterol or the presence of ergosterol as a detectable sterol (24). Critically, numerous protists that do not group on phylogenetic trees with fungi also synthesize ergosterol (25). These include trypanosomatid flagellates and *Euglena* (of the protist phylum *Euglenozoa*) (26–28), *Chlorella* and *Chlamydomonas* (Archaeplastida) (29, 30), and *Acanthamoeba* (Amoebozoa) (31). The taxon distribution (10) of this biochemical characteristic therefore suggests that utilization of ergosterol is at least as old as the major diversification of the eukaryotes, and consequently ergosterol cannot be used as a synapomorphy for fungi. It is therefore important that we no longer consider ergosterol as a useful biomarker for tracing fungi in environmental samples because this is not a reliable indicator of fungal presence/abundance (24). Even in most of the situations in which this biomarker has been used to detect fungi, i.e., plant and soil environments, nonfungal eukaryotes that produce ergosterol are found. As such, ergosterol detection should not be used as a biomarker specifically to quantify fungi without further checks on community composition such as environmental DNA diversity and abundance approaches, methods that essentially make the use of ergosterol detection as a proxy for fungal presence/biomass redundant.

The Fungal Cell Wall

Another trait thought useful for diagnosing fungi was the carbohydrate chemical composition of the cell wall. "Perhaps one of the most mutually satisfying by-products of fungal cell wall research, to taxonomists and biochemists alike, is the close correlation that can be established between chemical composition of the cell wall and major taxonomic groupings elaborated on morphological criteria," wrote Bartnicki-Garcia in 1968 (32). This work showed that many major taxonomic groups within the Fungi had different cell wall carbohydrate chemical compositions. This was cutting-edge research at the time and clearly demonstrated that the cell wall diversified in composition across the major fungal groups (see Table 1 in reference 32). As such, it was thought that these chemical characteristics were therefore diagnostic for higher taxonomic groups within the Fungi and not for the Fungi as a single group. Later work demonstrated that the chitin synthesis pathway has been lost in several fungal groups, for example, in *Pneumocystis* (33), an ascomycete fungus (34), therefore showing a clear example of secondary loss of a chitin cell wall. Indeed, numerous fungal groups have different cell wall compositions, while some fungi lose their wall (e.g., many fungal zoospores) or modify its components dependent on the life cycle stage (see reference 35 and Fig. 3 in reference 36).

Further work has shown that several groups initially classified as Fungi and thought not to have a chitinous cell wall (i.e., oomycetes and hyphochytriomycetes), but which were later shown to be members of the stramenopiles (37), do produce chitin in their cell walls (38, 39). Indeed, the ability to lay down chitin on the cell surface is present across numerous eukaryotic groups. These include, for example, *Entamoeba* (of the protist phylum Amoebozoa) (40, 41), *Trichomonas* (Excavata) (42, 43), Diatom and Pseudofungi (stramenopiles) (38, 44–46), *Plasmodiophora* (Rhizaria) (47), and *Chlorella* (Archaeplastida) (48). We note that in the case of the green alga *Chlorella* it has been suggested that the presence of a chitin synthesis pathway and a chitin cell wall is the result of horizontal gene

transfer (48). Yet even with this caveat, the taxon distribution of the production of chitin as a cell surface material suggests that this feature is older than the major radiations of the eukaryotes (47).

Considering the distribution of genes that encode chitin cell wall synthesis enzymes, it is clear that many of the features of chitin synthesis predate the radiation of fungi and are found across a wide diversity of eukaryotes. These include both chitin synthase (divisions 1, 2, and 3), chitinase (I and II), and chitin deacetylase enzymes (Fig. 1). These are collectively required to synthesize and remodel chitin polysaccharide. It is possible that horizontal gene transfer has led to this taxon distribution, but it is unlikely to have occurred on such a scale and across so many major divisions of the eukaryotes. As such, chitin as a metabolic trait and a feature of cell wall composition must have predated the radiation of fungi, therefore invalidating chitin synthesis and/or the chitin cell wall as a useful character for Fungi or any groups within.

β-Glucans are also key components of some fungal cell walls (e.g., 32, 35, 36). However, a range of β-glucans are found associated with cell wall structures in a number of protist groups, for example, associated with encystment of *Acanthamoeba* (Amoebozoa) (49, 50), the oocyst of *Toxoplasma* (50), and filamentous growth in the oomycetes (Stramenopiles) (39). Furthermore, the β-glucan paramylon can be produced at high quantities by *Euglena* (Euglenozoa) (51). Taken together, this demonstrates that β-glucan biosynthesis and utilization as a cell wall component predate the origin and diversification of fungi.

Both the β-glucan and chitin data demonstrate that the term "fungal cell wall" is a misleading concept and that it is actually a group of highly heterogeneous and dynamic traits (32, 35). These traits are found in many taxonomic groups that do not branch with or within the fungi; thus, it is important that we abandon this concept as a diagnostic feature.

NO SYNAPOMORPHY FOR THE FUNGI

The historical record of cellular and biochemical traits used as fungal synapomorphies summarized above demonstrates that the search for a diagnostic unifying character for the Fungi has failed. To paraphrase Tom

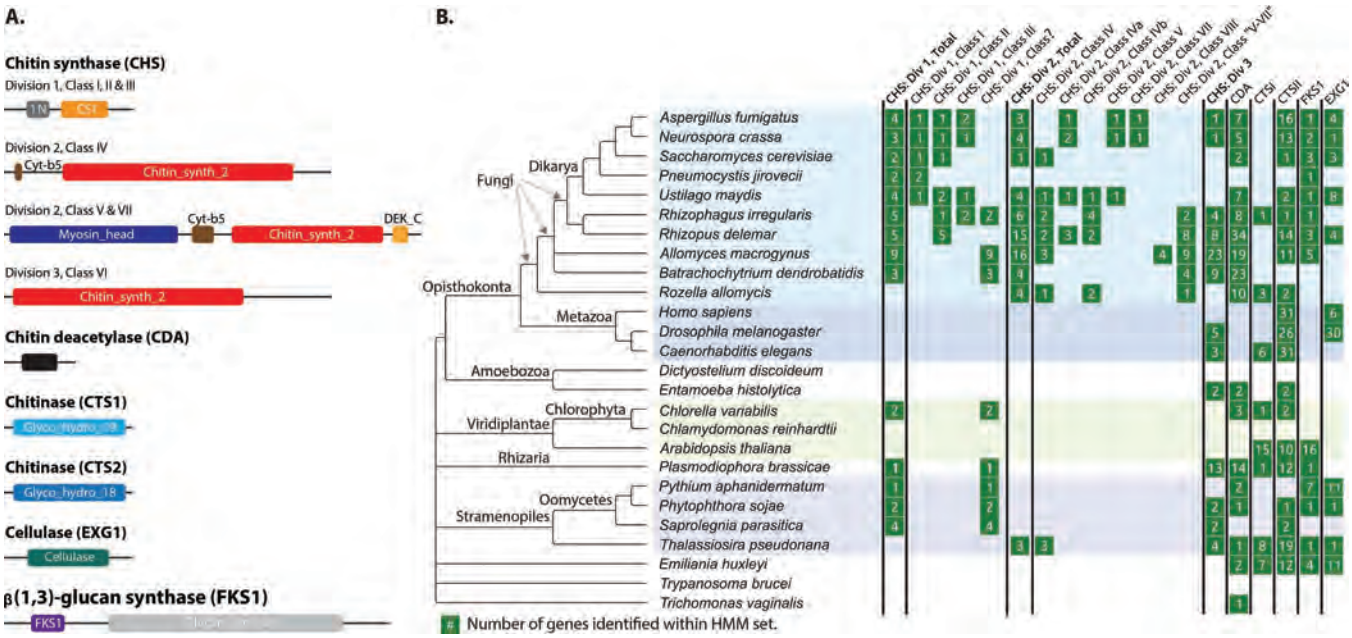


Figure 1 Diversity and distribution of gene families known to function in chitin cell wall synthesis or remodeling. (**A**) The diversity of domain architectures (as identified using PFAM [184]) for chitin cell wall synthesis or remodeling gene families. Note the gene fusion between a myosin head domain motor protein and a chitin synthase which was previously suggested to be fungus-specific (92). (**B**) The taxonomic distribution of putative homologues across a subset of eukaryotic taxa identified using a custom-built set of domain-specific hidden Markov models kindly provided by Jason Stajich and Divya Sain (185). The fungal component of the phylogenetic tree is based on Spatafora et al. (186).

Bruns, there is no fungal synapomorphy; get used to it (personal and public communication). And indeed, this is a state of affairs eloquently outlined by others (52), who coined the phrase "Bruns' law" to describe the situation of dissolving fungal synapomorphies. Yet the taxa that are indisputably fungal (e.g., Dikarya) form a coherent monophyletic group (e.g., 53–55) that undoubtedly represents a highly successful group of organisms both in terms of evolutionary diversity (e.g. 56) and ecological biomass. A more important question therefore is to ask what evolutionary transitions at or near the basal radiation of fungi tell us about the evolution, diversification, and success of this group.

EVOLUTION AT THE DAWN OF FUNGI AND THE RISE OF FUNGAL PHENOTYPES

Cavalier-Smith has set out a scenario for the origin of fungi from a protistan ancestor involving the loss of phagotrophy and the coupling of absorptive trophic function (also called osmotrophy) with cell wall development (often in the form of polarized hyphal growth) (57). Using phylogenetic reconstructions to infer characters deep within the fungal radiation, Cavalier-Smith also inferred that the ancestral fungus had an alternate nonflagellated trophic phase and a zoosporic phase (spore with a flagellum) (57). Later work involving phylogenetic reconstruction of the fungi with improved taxon and gene sampling provided data consistent with this hypothesis (53). Therefore, loss of a flagellum represents one or more derived transitions within the fungal radiation (53, 58). The loss of a flagellum is, for example, linked to radiation of terrestrial fungi, including the diversification of the Dikarya, which encompasses the majority of sampled fungal forms (53).

Interestingly, Cavalier-Smith's hypothesis for the origin of fungi involves changes in four linked biological/cellular functions, suggesting that these characteristic changes were synergistic. Specifically, these changes involved:

1. Loss of phagotrophy, which removed the requirement for a flexible cell surface membrane, allowing fungal cells to develop rigid and structurally reinforced cellular surfaces
2. Rigid reinforcement of the cell surface, which protects the cell against high turgor pressures, allowing cell function to flood the cytoplasm with high concentrations of solutes, which (i) allows for a very fast metabolic rate and (ii) allows a cell to assert high force to penetrate and ramify into robust structures as part of their feeding process
3. Evolution of rigid cells, allowing for polarized growth at the hyphal tip
4. A shift to external processing (digestion) of complex chemical compounds coupled to active transport of processed metabolites into the cell (osmotrophy or absorptive feeding [57])

Several emergent properties arise from the evolution of polarized, reinforced, osmotrophic functions. Fungi can essentially feed as they grow; i.e., they can couple the act of growth with nutrient sensing and metabolite processing, allowing for improved economy. These characters have made fungi very effective feeders, especially in environments composed of complex biological structures where fixed carbon, phosphorus, and nitrogen are packaged into recalcitrant materials which need to be broken down for utilization. This function is reflected by the observations that environments showing these characteristics, such as plant hosts, animal hosts, soil, sediments, and "litter," are often associated with high fungal biomass and diversity (4, 53, 56, 59–62). Although this pattern of evolution certainly occurred early in the radiation of the fungi, it is important to recognize that an evolutionary transition that included these four synergistic cellular/metabolic changes occurred on multiple occasions throughout the eukaryotic tree of life. For example, similar independent transitions occurred in the lineages that gave rise to the Pseudofungi (i.e., oomycetes and hyphochytriomycetes) and *Corallochytrium* (37, 63). These features are therefore not unique to fungi, yet we believe that studying how these features evolved in fungi and comparing them to other eukaryotes represents a fruitful path for understanding how fungi came to be and why they became such a successful and highly diverse group.

OSMOTROPHY RESULTS IN PUBLIC GOODS INTERACTIONS AND SYMBIOTIC ASSOCIATIONS

As a consequence of the fungal osmotrophic function, nutrients are processed in the extracellular environment, a function that underpins how some fungi operate both as parasitic and/or mutualistic symbionts of other organisms. This function has been especially important in how fungi form diverse and critical associations with land plants (64–68). As a result of the loss of phagotrophy and diversification of osmotrophic functions, fungi must also engage in public goods interactions (e.g., 64, 69–71). This is because metabolites processed by secreted enzymes outside the cell are essentially rendered accessible to any other organism that

occupies the same environment and can take up and make use of the metabolites released (discussed in references 72, 73). As such, communities where fungi function are also the sites of public goods competitions.

Microbes evolved many strategies to maximize public goods competitions. In fungi, two common biological features are predicted to function to maximize osmotrophic public goods functions. First, growth as multicellular hyphal networks allows the colonization of three-dimensional space, which hypothetically acts to minimize loss of metabolites to competitors. Indeed, comparative experiments have demonstrated that public goods functions drive the evolution of multicellularity in yeast to maximize utilization of hexose sugars released by the function of secreted enzymes (70). It has also been shown that hyphal networks allow fungi to explore wider environments, accessing additional nutrient sources where such sources are patchy, i.e., separated by zones where nutrients are absent. Fungal networks therefore demonstrate the properties of high transport capacity. Importantly, these features are also enhanced by larger networks, where the relative cost of such development is reduced by increase in the size of the network formed (74). It remains to be tested if the development of a hyphal network provides adaptive advantages over nonnetworked competitors in terms of maximizing specific osmotrophic function and public goods competitions (such as breakdown of recalcitrant material). However, as Bebber et al. (74) demonstrate, hyphal networks do maximize the economics of growth in many environments in which fungi are both diverse and abundant.

Second, fungi have hugely diversified secondary metabolism (e.g., 75). Many of the products of this metabolic function act as antimicrobial toxins (76), and many of these secondary metabolic properties are encoded by clusters of genes (e.g., 77–81). Examples of these clusters encode both metabolic pathway components and transporters (e.g., 82) to tightly couple expression (e.g., 83) and chromosomal linkage (and therefore coinheritance) of the genes encoding biosynthesis, export, and detoxification. The role of these metabolic functions is often unclear. Yet synthesis and secretion of many toxins is likely to function to exclude microbes that do not possess a cognate gene cluster encoding detoxification phenotypes from an environment where a public good is present and the toxin is produced. Such traits, often described as "spiteful" in the evolutionary ecological literature, therefore act to police access to the public goods released by osmotrophic functions (72, 73).

Even though filamentous growth, construction of a robust cell wall, loss of phagotrophy, and osmotrophic feeding make up the fungal lifestyle, and underpinned the success of fungi, it is important to note that none of these features are diagnostic for the kingdom. This is because they are all found together elsewhere on the tree of life (e.g., 37, 63). Our current understanding of both fungal diversity and biomass in terrestrial environments, however, tells us that fungi are particularly good at performing these functions. In the next section, we seek to understand what underpinned the success of this lifestyle.

WHY ARE FUNGI ECOLOGICALLY SUCCESSFUL?

Older than Their Competitors

As argued by other authors (e.g., 57), the relative age of the fungi compared to other fungus-like groups, e.g., oomycetes (Pseudofungi), hyphochytriomycetes (Pseudofungi), and *Corallochytrium* (Holozoa, Opisthokonts), may explain why they dominate many environments compared to other eukaryotic groups that grow and feed in a similar manner. Put simply, the fungi "got there first" and have successfully retained such niches by further adaptation that underpins the competitive exclusion of others. Molecular clock data suggest that the Dikarya, one of the major radiations of the terrestrial fungi, seem to date to ~500 million years ago (84). In contrast, the major radiation of the oomycetes, a group that also successfully couples a rigid cell wall, polarized cell growth, and osmotrophic feeding, seems to have occurred ~250 million years ago. The largely plant-associated Peronosporales are estimated to date from ~150 million years ago (85), some considerable time after fungal forms colonized similar plant-associated habitats. Such hypotheses (57) are difficult to test, but it is likely that fungi have had an extended time frame in which to evolve capabilities to maximize retention of terrestrial niches such as plant-associated environments.

Economic Coupling of Growth and Feeding

One other possibility is that fungi are just better at coupling rigid cell wall synthesis, polarized cell growth, and osmotrophic feeding (i.e., feeding as they grow) than their nonfungal competitors. By "better," we mean they can perform this function at a higher rate and/or with greater metabolic efficiency, allowing them to outcompete others. If this is the case, then it is likely due to adaptations in cytoskeletal functions that allow high-speed trafficking and efficient ordering of vesicles and other cellular machinery which drive rapid/efficient hyphal tip growth and feeding (such machinery

is discussed below). Indeed, some data suggest that filamentous ascomycetes can achieve very fast rates of vesicle transportation to growing hyphal tips (86). Furthermore, many fungi possess a range of unique endomembrane/cytoskeletal systems associated with the center of tip growth. These include, for example, the cytological characteristic of a vesicle organizing center called the Spitzenkörper and/or the apical vesical crescent, found at the hyphal tip of many fungi, which identifies the main area of growth in filamentous Dikarya and zygomycetes, respectively (e.g., 87, 88). Other non-Dikarya fungi have also been shown to have hyphal tip structures similar to the Spitzenkörper, specifically *Allomyces*, that branch among the deepest phylogenetic branches within the fungi (89). Yet these systems are absent in many fungi, and where present, the Spitzenkörper is highly variable in cellular structure (e.g., 87, 88). As such, it is hard to identify a cellular trait that specifically defines polarized cell growth across the Fungi.

Investigating how fungal and nonfungal microbes couple rigid cell wall synthesis, polarized cell growth, and osmotrophic feeding, however, is likely to be important for understanding the evolution of characters that underpinned the success of the Fungi. For example, a gene fusion between the actin cytoskeleton myosin motor head domain and a division 2 chitin synthase domain (e.g., 90, 91) was proposed to be a "unifying" character for the Fungi (92). This gene is localized to both vesicles and the hyphal tip and is involved in tip growth in *Ustilago* (93, 94). The myosin-chitin synthase gene fusion seemed like a good candidate for a fungal synapomorphy because the function of these protein domains involves cytoskeletal trafficking and cell wall synthesis and thus is directly implicated in polarized cell growth and rigid cell wall development. However, it is important to note that this character is absent in many established fungal groups (e.g., *Saccharomyces* and *Schizosaccharomyces* [90]) (Fig. 1), though likely due to secondary loss. Furthermore, since gene fusions are often unstable characters undergoing both fission, horizontal gene transfer, and domain rearrangement (95–97) (which has also been demonstrated in fungi [98]), it is unclear if synapomorphies of these types can be trusted. Indeed, there is some evidence that the myosin-chitin synthase gene fusion may not be specific to fungi and is present in other opisthokont lineages (63, 91) and therefore is invalid as a synapomorphy for the Fungi. However, even though it is not a unifying feature, the acquisition of the myosin-chitin synthase gene fusion undoubtedly contributed to filamentous growth functions within the Fungi.

Looking elsewhere in fungal growth and feeding may also prove fruitful for understanding what functions underpinned the success of fungi. Cellular growth in fungal filamentous structures occurs almost exclusively from the tip (99); this is part of the function that allows them to grow as they feed. This feeding process occurs at a high rate in Pezizomycotina that specialize in filamentous growth (86). However, these endomembrane and cytoskeletal systems were co-opted to control bud growth in *Saccharomyces cerevisiae*. Subsequently, *S. cerevisiae* has been developed as a model system for studying protein function associated with polarized growth (99, 100) (Fig. 2).

Briefly, polarized growth occurs by delivery of secretory vesicles from the Golgi (Fig. 2A, see step i) along cytoskeletal tracks to predetermined areas of the cell membrane (in filamentous fungi this would be at the hyphal tip). This process is initiated by Cdc42, which is activated by its guanine-nucleotide exchange factor Cdc24 (Fig. 2A-ii), which leads to the development of a network of physically interacting proteins (99, 100) known as the polarisome (Fig. 2A-iii). The polarisome, and specifically the formin Bni1, radiates actin cables (101) which play a key role in exocytosis (102). Msb3 and Msb4 interact with Spa2, a scaffold protein of the polarisome (Fig. 2A-iv), and are hypothesized to recruit Cdc42 from the cytosol at the site of tip growth (103). Post-Golgi secretory vesicles are transported along the actin cables using a type V myosin (Myo2) motor protein (104, 105) (Fig. 2A-v) to dock with another multiprotein complex called the exocyst in a process that is dependent on Sec4 and its guanine-nucleotide exchange factor Sec2 (106, 107) (Fig. 2A-vi). This process therefore enables the vesicle to be guided to its target site on the plasma membrane (108). Cdc42, along with Rho1, is required for localization of Sec3, which acts as a spatial marker for the formation of the exocyst (Fig. 2A-vii), while Rho3 and Cdc42 mediate docking of the Golgi-derived vesicle (Fig. 2A-viii). In filamentous fungi, homologous sets of proteins function similarly; however, an even more complex set of polarity factors are present; e.g., in addition to Cdc42, another RhoGTPase, Rac1, and an expanded set of effectors (guanine-nucleotide exchange factors and GTPase-activating proteins) play key roles in regulating these processes (109–111).

The polarisome and the exocyst, and proteins associated with these complexes, are very important for establishing the temporal and spatial control of polarized cell growth. They also fit the criteria of key functional adaptations that underpin the success of fungi as microbes because their function is linked to rigid cell wall

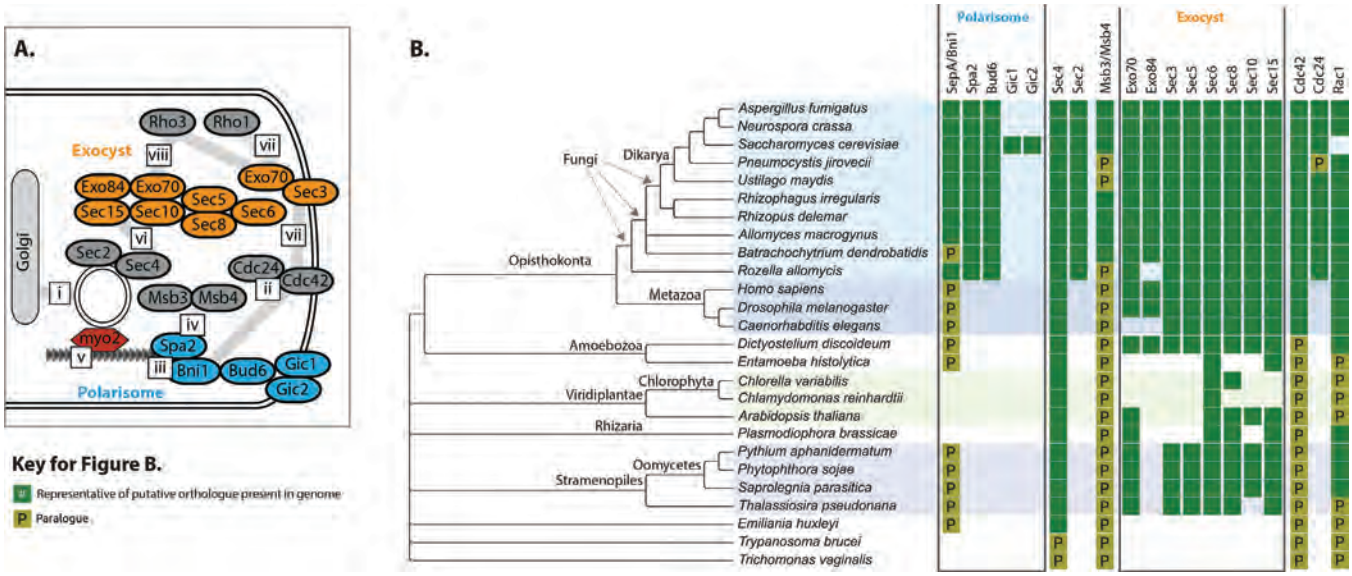


Figure 2 Diversity and distribution of gene families known to function in hyphal growth. (**A**) A cartoon illustrating how proteins interact relating to subprocesses which govern vesicle trafficking associated with hyphal growth. Functions are briefly discussed in the main body of this manuscript and are marked i to viii. (**B**) The taxonomic distribution of putative orthologues identified using reciprocal BLAST searches and phylogenetic methods across a subset of eukaryotic taxa (data not shown). The fungal component of the phylogenetic tree is based on Spatafora et al. (186).

development, polarized cell growth, and osmotrophic feeding. As such, these are examples of the systems we should investigate from the perspective of fungal cellular function and GTPase-activating protein evolution.

Comparative genome analysis shows that the exocyst system and Sec4 orthologues are conserved across a diversity of eukaryotes (112, 113), suggesting a broad function in directing the interaction between vesicle, membrane, and cytoskeleton (Fig. 2B). Given current genome sampling, polarisome components seem to be unique to fungi. However, preliminary genomic data suggest that these components could be present in unpublished but publicly available opisthokont protists (data not shown), thus demonstrating further evidence that many functions and gene networks associated with fungal growth are not specific to fungi.

INCREASED SAMPLING FURTHER CHALLENGES OUR UNDERSTANDING OF THE TREE OF LIFE

So far, we have reviewed the data demonstrating that, to date, no cellular or genomic characters have been identified that represent a synapomorphy for the Fungi. As a consequence, it may be argued that the best definition of fungi is a phylogenetic definition. Specifically, our best approximation of defining the kingdom is declaring, "Phylogenetic cluster X incorporating known taxa A, B, C, etc., represents the Fungi." This is a challenging state of affairs because where one draws the line on a phylogenetic tree to define the kingdom is also problematic. This is because the phylogenetic tree is incomplete in terms of genetic data and the diversity of groups sampled. Several discoveries over the past 20 years have repeatedly challenged how and where we mark the Fungi on a phylogenetic tree. In the next section we will briefly outline these discoveries.

Protistan Relatives of Fungi

Multigene phylogenetic analyses have demonstrated that the opisthokont radiation is composed of two major evolutionary branches: (i) the Holozoa, which comprise Metazoa, Choanoflagellata, Filasterea, *Corallochytrium*, and Ichthyosporea, and (ii) the Holomycota, which comprise fungi, *Fonticula*, and *Nuclearia*, with *Fonticula* and *Nuclearia* forming a robustly supported clade sister to fungi (63, 114, 115). This branching relationship suggests that *Fonticula* and *Nuclearia* should have characteristics similar to fungi and to the exclusion of others including the Holozoa. In reality, however, what we see is a diverse mosaic of characters, initially attributed to fungi and now identified to be

present across the opisthokonts, specifically in Holozoa protists.

Holozoa comprise a wide diversity of biological forms, which are not possible to discuss at length here. However, for the purposes of this article, it is important to mention that representatives of the nonmetazoan Holozoa show many of the characteristics thought to typify fungi. These include *Corallochytrium limacisporum*, a free-living protist which appears to have lost phagotrophy and feeds via osmotrophic function, a pattern of convergent evolution mirroring that which occurred within or at the base of the Fungi (63). Similarly, during host-associated stages, holozoan parasites *Dermocystidium salmonis*, *Ichthyophonus hoferi*, *Psorospermium haeckii*, and *Rhinosporidium seeberi* absorb nutrients through their cell wall structures (for review, see reference 116). Members of the Ichthyosporea (e.g., *A. parasiticum* and *I. hoferi*) develop polar growth similar to that seen in fungal hyphae (63, 117). Representatives of the Ichthyosporea also showed diversified sets of chitin synthase genes (63) presumably used as cell wall components. *I. hoferi*, for example, has chitin in its cell wall (118), and the Dermocystida *R. seeberi* appears to encode a class 2 chitin synthase (119). *C. limacisporum* putatively encodes a division II class IV/V/VII chitin synthase-myosin fusion (63) previously thought to "unify" the Fungi (92). Furthermore, *Corallochytrium* and members of the Ichthyosporea have a developmental cycle that involves a single-celled flagellated zoospore (e.g., *Sphaerothecum destructans*) and a multinucleated (coenocyte) feeding structure, a developmental cycle that has similarities to that of "chytrids" (i.e., fungi that form spores with a flagellum [zoospores] and which were at one stage classified as Chytridiomycota but were later divided into three phyla: Chytridiomycota, Blastocladiomycota, and Neocallimastigomycota [63, 116, 120, 121]). Both Ichthyosporea and *Corallochytrium* branch on the Holozoa side of the opisthokont tree, but current phylogenetic analysis does not confirm with strong support whether these groups are monophyletic (63), making it difficult to reconstruct the evolution of these "fungal-like" characteristics in the Holozoa as either multiple cases of convergent evolution or retention of ancestral traits.

Nuclearia are a group of pseudopodia/filipodia-forming "obligate" amoebae and were first identified as a separate protist lineage within the Opisthokonta by small-subunit (SSU) rDNA sequencing and phylogeny (122). The related *Fonticula alba* is a cellular slime mold and an amoeboid protist which can form multicellular fruiting bodies (115, 123). Both of these taxa are phagotrophic and lack a trophic phase typified by absorptive/osmotrophic feeding and a chitin cell wall (115); as expected, they fail many definitions of fungi (e.g., 57). The phylogenetic placement of *Fonticula*/*Nuclearia* as a close relative of fungi (e.g., 63, 115) separates the Fungi from many holozoan protists which also have fungus-like characteristics (discussed above). Furthermore, this placement suggests that a close ancestor of fungi was a pseudopodia/filipodia-forming phagotrophic cell with an alternative flagellated life cycle stage.

Interestingly, environmental sequencing has also suggested a large diversity of phylogenetic branches associated with *Fonticula* in both freshwater and marine environments. These results suggest a wealth of unexplored biodiversity associated with this branch (124, 125), which could potentially further confuse evolutionary trait reconstruction at or near the origin of fungi.

New Branches Near the Origin of Fungi

The history of our understanding of fungal diversity has been dominated by what can be cultured on selective solid media (e.g., 126). Such methods have proved very effective at recovering some fungal groups, especially Dikarya, but are less effective at recovering other groups such as the chytrids and non-Mucorales zygomycetes. Microbes that take part in complex symbiotic associations with other organisms or are propagated in liquid environments are often excluded by culture plate sampling techniques, meaning that our understanding of biodiversity is largely incomplete. Culture plate methods also act to select for certain fungal groups that grow quickly on solid media with abundant nutrients. This means that many fungal groups can readily be recovered from natural environments even though these microbes were not active or abundant in the initial environment sampled. Their rapid growth on culture plates means they competitively exclude those microbes that were actually active and dominant in the environment sampled. Therefore, such sampling can be positively misleading regarding the true diversity of fungi, and indeed other eukaryotic microbes, present in an environment. As such, our understanding of the tree of life is partial and biased (as discussed in references 127, 128).

Our understanding of how extant biodiversity maps onto the tree of life, specifically at the base of major groups such as fungi, has been challenged by two lines of research. First, environmental DNA analysis has revealed that uncultured groups, which are both diverse and abundant in natural environments, represent substantial additions to the tree of life in terms of both branch placement and molecular diversity (e.g., 128–130). These include, for example, a group formally

known as soil clone group 1 (131), which has been detected in numerous soil environments (132) and which was later classified as Archaeorhizomycetes within the ascomycete subphylum Taphrinomycotina (133). Second, molecular sequencing combined with phylogenetic analysis of groups once thought to be distant relatives of fungi has shown that such groups branch within, or at the base of, the fungal radiation (e.g., microsporidia, discussed below).

There are now examples of both types of these newly discovered and placed groups that collectively challenge where we draw the line that defines fungi and consequently which characters are used for this definition. In the following section we shall briefly introduce some of these groups and what data are available pertinent to our understanding of the origins and classification of fungi. In many cases the phylogenetic placement of these groups relies on (partial) 18S SSU rDNA gene phylogenetic analysis and also includes rDNA sequences that form long branches in phylogenetic trees. Phylogenetic analysis of such datasets provides very inconsistent and often misleading results (134–136), meaning that the placement of these groups relative to each other and the boundary that defines fungi is largely unresolved and requires comprehensive multigene phylogenetic analysis.

Aquatic Environmental Sequencing and the Expansion of the Chytrid Branches

The use of environmental DNA methods (127) for sampling aquatic environments, including both freshwater and marine environments, has revealed a diversity of novel SSU rDNA sequences that cluster among the deepest branches of the Fungi closest to known chytrid taxa (e.g., 137). The phylogenetic placement of these sequences often involves relatively long branches, and the bootstrap support is often weak; for this reason, the phylogenetic relationships reported should be treated with caution. Nonetheless, these data suggest a diversity of undescribed and uncultured chytrid-like forms that exist in aquatic environments (e.g., 137–141). Strikingly, these sequences show a trend of low levels of sequence similarity to taxonomically described fungi compared to environmental sequences that cluster with the Dikarya (142), suggesting there is a wealth of unexplored phylogenetic diversity present in aquatic environments and branching within or close to known chytrid groups. However, it is possible that this pattern is exaggerated by a slower rate of rDNA variation in Dikarya as compared to the chytrids.

It has been hypothesized that this chytrid diversity is an important component of the heterotrophic flagellate community in water column communities, playing a key role in the microbial loop as either parasites and/or saprotrophs (143–145). However, the nature of the detection of these groups (environmental sequencing) means that understanding their biology and ecology is difficult. However, clear examples of chytrid groups (146, 147) and their affiliates (145) have been identified associated with diatoms, suggesting trophic interactions with primary producers in both marine and freshwater environments. Their phylogenetic placement as allies to the Chytridiomycota and other chytrids would, however, suggest that they have a life cycle with alternating nonflagellated trophic and zoosporic phases and can form feeding structures reinforced with a chitin cell wall. However, work is needed to investigate the biology of these groups to confirm the presence of these life cycle characteristics.

Possible Fungal/Chytrid-Like Group Known as Basal Clone Group 1

Among the diversity of SSU rDNA sequences sampled from aquatic environments, one distinct group has consistently been identified. This group of sequences was initially detected in marine sediments (148) and deep-sea environments (139, 149, 150) using PCR targeting a wide diversity of eukaryotic SSU rDNA combined with clone library selection and sequencing (see GenBank sequence KD14-BASS [GenBank accession: EU154992] or DSGM-63 [AB275063] for exemplar representatives of this group). This clade has subsequently been detected in RNA and DNA samples from a range of marine environments (e.g., 141, 142), including both sediments and water-column samples, and is associated with both large and small filtration fractions, suggesting that this group has a complex life cycle in which it forms different cellular morphotypes or interacts with second-party cells (141). Phylogenetic analysis of the SSU rDNA sequences gives a mixed picture of the placement of this group, forming a relatively long and distinct branch that in some analyses is placed among the Fungi (e.g., 149), while others show it as a branching sister to Ichthyosporea (e.g., 139). Work is needed to assess the ecological roles and evolutionary position of this diverse and divergent group. Such analyses will likely have implications for understanding the evolutionary patterns within the Opisthokonta independent of whether or not this group branches within Fungi.

Microsporidia

Microsporidia have historically been placed at numerous branching points in the tree of life, and their lack of many eukaryotic cellular structures (e.g., flagella,

mitochondria, and typical stacked Golgi) has made it difficult to assign them to a higher taxonomic group based on ultrastructural data (57). SSU rDNA phylogenetic trees initially placed the microsporidia at the base of the eukaryotic radiation. This result, along with the absence of these cellular structures and the characteristic of short prokaryotic-like SSU rDNA genes, had been used to argue that they were derived from an early—premitochondrial—phase of eukaryotic evolution (151). This hypothesis has been refuted with the demonstration that microsporidia possess highly reduced mitochondria-like organelles (152) and that microsporidian nuclear-encoded proteins are of mitochondrial ancestry (153, 154). Additionally, phylogenetic analysis of nuclear-encoded protein coding genes with appropriate correction for site rate variation has shown that the microsporidia branch with fungi (e.g., 155, 156). Gene synteny analysis of the *sex*-related locus demonstrated that fungi and microsporidia share a unique syntenic pairing of the RPS9 and RPL21 encoding genes, supporting the close phylogenetic association of fungi and microsporidia (157). The placement of microsporidia with fungi therefore demonstrates that the microsporidia are a striking example of genomic and cellular reduction when compared to their fungal and protist relatives (158).

The microsporidia encompass over 1,500 described species, which are all intracellular parasites that infect other eukaryotes. The defining feature of microsporidia is the development of long polar tubes coiled within the parasite's cell, which are used to infect the host cell (158) as part of their life cycle, although some microsporidia can invade host cells via host phagocytosis (159). Microsporidia do not form a walled trophic form and they do not form filamentous structures similar to hyphal cells (57), which are characteristics argued to underpin the successful radiation of the fungi discussed above. The majority of microsporidia do form a chitin cell wall around their spore (57). Because microsporidia lack flagella, they do not form an alternate nonflagellated trophic phase and a zoosporic phase as part of their life cycle. Such characteristics are thought to typify the ancestral fungus (discussed above) and are found in *Rozella* (discussed below). As such, independent of where the microsporidia are placed relative to the origin of the fungi, they have secondarily lost these characteristics.

The pattern of genomic and cellular evolutionary reduction that is evident in the microsporidian lineage makes it difficult to identify characters that can be putatively assigned as shared synapomorphies with established fungal taxa, which in turn further complicates how we define the kingdom. Specifically, it makes it very difficult to support either of these statements: "Microsporidia belong in fungi," or alternatively, "Microsporidia belong as a sister group to fungi," even though improved multigene phylogenies suggest that they branch with *Rozella* (55) and *Rozella* has historically been classified as a fungus (e.g., 160).

Cryptomycota-Rozellomycota-Rozellida-Rozellosporidia

A range of phylogenetic analyses suggest that *Rozella* represents the first phylogenetic branch among fungi (53, 55). This conclusion is based on the premise that *Rozella* has historically been classified as a fungus (e.g., 53, 121, 160), although the treatment of *Rozella* as a fungus is now debated (161, 162). As such, the biology of *Rozella* and associated groups is very important for understanding the evolutionary transitions at, or near, the base of fungi. Concatenated multigene phylogenetic analysis suggests that *Rozella* branches with the microsporidia, although alternative branch placement with statistical comparisons could not reject microsporidia branching separately from *Rozella* as the next branch below the fungi (55). Shared derived horizontal gene transfers (HGTs) were also identified that support the sisterhood of *Rozella* and the microsporidia (55, 163), but again the taxon distribution of these characters could potentially be amended by additional genome sequencing demonstrating a wider distribution of the HGTs.

Rozella has also been shown to be part of a large and diverse group of uncultured microbes, primarily identified through environmental DNA techniques (and which also seem to form associations with diatoms [147, 164, 165]). This environmental group, along with *Rozella*, has been given a range of different names, i.e., Cryptomycota, Rozellomycota, Rozellida, and Rozellosporidia. *Rozella* (unlike microsporidia) has a life cycle composed of alternating nonflagellated trophic and zoosporic phases (166). Fluorescent *in situ* environmental DNA methods combined with antibody staining of microtubules demonstrated that some subgroups within the environmental clade related to *Rozella* also develop alternative nonflagellated and zoosporic phases (165). These results are consistent with this biphasic life cycle being present in the earliest diverging groups of the Fungi.

Like microsporidia, *Rozella* and its uncultured relatives were thought to lack a cell wall during trophic growth within the host (160). Indeed, *Rozella* grows within its host as naked unicellular thalli (160). Furthermore, *Rozella* is thought to feed phagotrophically on host cellular structures because it was shown

to form pseudopod-like projections surrounding host cytoplasm (167) during infection. Fluorescent *in situ* RNA labeling combined with cell wall staining of cells captured using environmental sampling allowed analysis of only fragments of the life cycle of additional representatives of this group but suggested that a chitin cell wall is absent from a subset of life cycle stages, although it should be noted that such sampling is incomplete either for the life cycle of an individual species or across the wider group (165). Follow-up work has since demonstrated that *Rozella* does indeed produce a chitin cell wall as a resting spore as well as during the invasion process, where the production of a cell wall is specifically linked to host penetration (55, 92), but it does not seem to use a cell wall during trophic growth. This pattern of development has similarities with how microsporidia use their chitin cell wall. Consequently, *Rozella*, like some true fungal groups (discussed above), fails the definition of fungi that requires a developmental stage composed of a trophic form with a chitin cell wall (57).

Recent exciting work has shown a number of established cultures of intracellular microbes branching within the radiation of environmental sequences that branch with *Rozella* (168). This allows us to further investigate the phylogeny and cellular characteristics present in this otherwise cryptic environmental group. One of the striking results of this work is the demonstration that subgroups within this radiation possess cellular structures similar to the microsporidia. Specifically, *Paramicrosporidium* was shown to produce unflagellated chitin and/or cellulose-walled spores (as indicated by calcofluor white staining) and grow within the host as unwalled nonphagotrophic meronts (168)—again like microsporidia. *Nucleophaga amoebae*, a microbe initially described in 1895 (169; see 170) as a "chytrid" fungus, was also shown to branch within the radiation of environmental sequences that branches with *Rozella* and, also like *Paramicrosporidium*, produces unflagellated chitin and/or cellulose walled spores (168, 170). Interestingly, *Nucleophaga* (see Fig. 1C and D in reference 170) and *Paramicrosporidium* (see Fig. 1C–E from reference 168) also develop putative anchoring disks and an atypical polar filament like microsporidia.

Haag et al. (171) identified a novel gut microbe of *Daphnia* named *Mitosporidium daphnia*. Genome sequencing combined with phylogenetic analysis showed that this taxon branched at the root of the microsporidia in a phylogeny of 53 gene families. However, rDNA phylogenetic analysis encompassing much wider taxon sampling shows that *Mitosporidium* branches within the radiation of environmental sequences that branch with *Rozella* (170). Cytological analysis shows that *Mitosporidium* possesses a microsporidia-like polar tube and lacks a flagellum for spore dispersal. Collectively, these results show, in comparison to what we know about *Rozella*, that this group seems to be a mosaic of fungal chytrid and microsporidian cellular features. This mosaic pattern of features is further complicated by evidence that representatives of this group (e.g., *Nucleophaga* and *Rozella*) can form pseudopodia-like cytoplasmic projections and possibly perform phagotrophy (167, 172), a feature that, under some definitions, must be absent for a group to be classified as fungi (57).

Aphelids

Aphelids are amoeboflagellate parasites of microalgae (173, 174) classified as protists. They form zoospores resembling those of chytrids, yet in contrast to known chytrids, aphelids feed via phagotrophy when inside their host (e.g., 173). *Amoebaphelidium protococcarum* (173), like other aphelids (175, 176), attaches to host cells, produces a cyst cell wall, and penetrates the host via a germ tube, a process that is similar to how *Rozella* and microsporidia enter the host. Once inside the host, they form pseudopods and feed on host-derived cytoplasm via phagotrophy, unlike microsporidia but possibly in a manner similar to the intracellular pseudopodia development observed for *Rozella* (167). Phylogenetic analysis based on three rDNA encoding genes and two protein encoding genes demonstrates strong support for the monophyly of microsporidia, *Rozella*, and the aphelids, which together are argued to form a sister clade to the fungi (173). These results were used, along with similarities in germ/infection tube apparatuses and a posterior vacuole, to suggest that these groups form a distinct monophyletic group (named aphelid-*Rozella*-microsporidia) (173) now formally classified by some as Opisthosporidia (162).

The data discussed in this section cover numerous groups newly and/or only partially sampled and demonstrate collectively that we are some distance from achieving an accurate census of the diversity of microbial forms that branch within, or close to, the Fungi. Furthermore, these data also show that we still know relatively little about the diversity of cellular and genomic features that are evident within groups that branch close to the Fungi. This state of affairs makes it very difficult to understand the evolution of traits such as cell wall development, hyphal growth, flagellum formation, phagotrophy, and pseudopodia formation across the deep branches of the Fungi. However, further investigation into these deep-diverging lineages

of fungi/protists will undoubtedly aid our understanding of the traits present in early fungi and help to explain the general ecological and evolutionary success of the fungal lineage.

PSEUDOPODIA FORMATION ACROSS THE DEEP BRANCHES OF THE FUNGI FURTHER CONFUSES THE FUNGI-PROTIST DISTINCTION

One current suggestion is to go against the historical classification of *Rozella* and *Nucleophaga* as fungi and exclude *Rozella* and its phylogenetic allies from the Fungi because they are thought to be phagotrophic (161). *Rozella* and related groups have been shown to form pseudopodia-like projections (167, 170), cellular features linked to phagotrophic function (172). Phagotrophy is argued as necessarily absent in taxa within the Fungi (161). However, it has been known for some time that chytrid zoospores from groups unambiguously classified as fungi also form short pseudopodia and develop amoeboid stages, e.g., *Amoebochytrium*, *Caulochytrium*, *Catenaria*, and *Paraphysoderma* (see 172). However, in these cases, pseudopodia appear to be used to crawl, and true phagocytosis, to our knowledge, has never been reported.

Pseudopodia (and the similar cellular structures of filopodia) are fine actin-based membrane-cytoplasmic cell surface projections used for both environmental sensing and cell motility and form structures necessary for phagotrophy. This cellular characteristic is found throughout the eukaryotes and is thought to have evolved early in their evolution (177), long before the emergence of the fungal lineage. In recent work, phylogenomic comparisons of genes encoding actin-based cellular protrusion proteins showed these gene families to be present in fungi (178). These include, for example, the Arp2/3 complex involved in actin-remodeling (179), which in metazoans is regulated by WASP and cortactin function (180). The seven subunits of this complex are present in all major eukaryote groups and almost all eukaryotes surveyed which make pseudopodia/filipodia, consistent with the idea that pseudopodia/filopodia development has a deep origin within the eukaryotes (178). In contrast, Ena/VASP, an actin-regulation protein which functions in pseudopodia/filopodia formation (181), is exclusive to Amorphea/Unikonta and appears to have been secondarily lost in the Fungi (178), although genome sampling in this study only included Dikarya and two chytrid genomes.

Recent and exciting work by Fritz-Laylin and colleagues has investigated the evolution of the genes encoding proteins with what they define as "α-motility" (motion driven by actin-filled pseudopods at the leading edge of the cell and involving fast migration and weak nonspecific adhesion to external surfaces [182]). Using comparative genomics, they showed that lineages with both WASP and SCAR/WAVE, activators of branched actin assembly, make the three-dimensional pseudopods needed for α-motility. These genes encode proteins responsible for "nucleation promoting factors" that drive assembly of branched actin by the Arp2/3 complex and are distributed throughout the eukaryotic tree, suggesting an ancient origin and function (178, 182). The distribution pattern of WASP and SCAR/WAVE encoding genes was then used to identify pseudopod function in the chytrid fungus *Batrachochytrium dendrobatidis*. Specifically, they demonstrated that zoospores, which naturally lack a cell wall, form *bona fide* dynamic pseudopod-like protrusions, and staining of these structures demonstrated a "shell of cortical actin surrounding the cell body and a dense network of filamentous actin filling the pseudopod" (182). To test this observation further, drug treatment with an actin nucleation inhibitor significantly reduced the number of cells with identifiable pseudopodia-like structures (182). These data confirm that species of established fungal taxonomic groups form pseudopodia-like structures.

It is therefore clear that the cytoskeletal systems associated with pseudopodia, a cellular function that overlaps with phagotrophic function, were not cleanly lost at the base of the Fungi and that elements of these cellular systems are retained within some fungal branches. As such, the hypothesis that loss of phagocytosis is the defining trait for the Fungi requires further work to understand the evolution of the cellular systems that drive this function. We suspect that even with a clear set of data identifying cell/molecular traits associated with phagotrophy, and a full understanding of how the genes that encode this function are distributed in fungi and protists, no clean separation between fungi and protists will be evident for this trait. This is partly because phagotrophic function, as we currently understand it, is defined by few specific protein families (e.g., 183). Furthermore, if such phagotrophic genes can be found, they are likely to have also been lost in other eukaryotic groups that have lost phagotrophy, for example, plants and oomycetes, making the loss of such traits an unreliable demarcation for the Fungi.

CONCLUSION

This review has discussed how the definition of the Fungi, and the list of taxa that are included within this

kingdom, have been in a continual state of flux. An additional purpose of this historical review of failed fungal synapomorphies is to demonstrate that cellular, molecular, and genetic characters are hostages to fortune; i.e., they are very likely to be shown by further sampling to have complex evolutionary histories which invalidate them as diagnostic characters. This is because the features that have been argued to define the kingdom are either only partially present in the Fungi or, more importantly, are present elsewhere in the tree of life (summarized in Fig. 3). In most cases, for features used to identify the kingdom Fungi, both situations are true. It can be argued that the loss of a trait within a monophyletic group, for example, the loss of ergosterol within the Fungi, does not invalidate the use of such a character as a defining synapomorphy

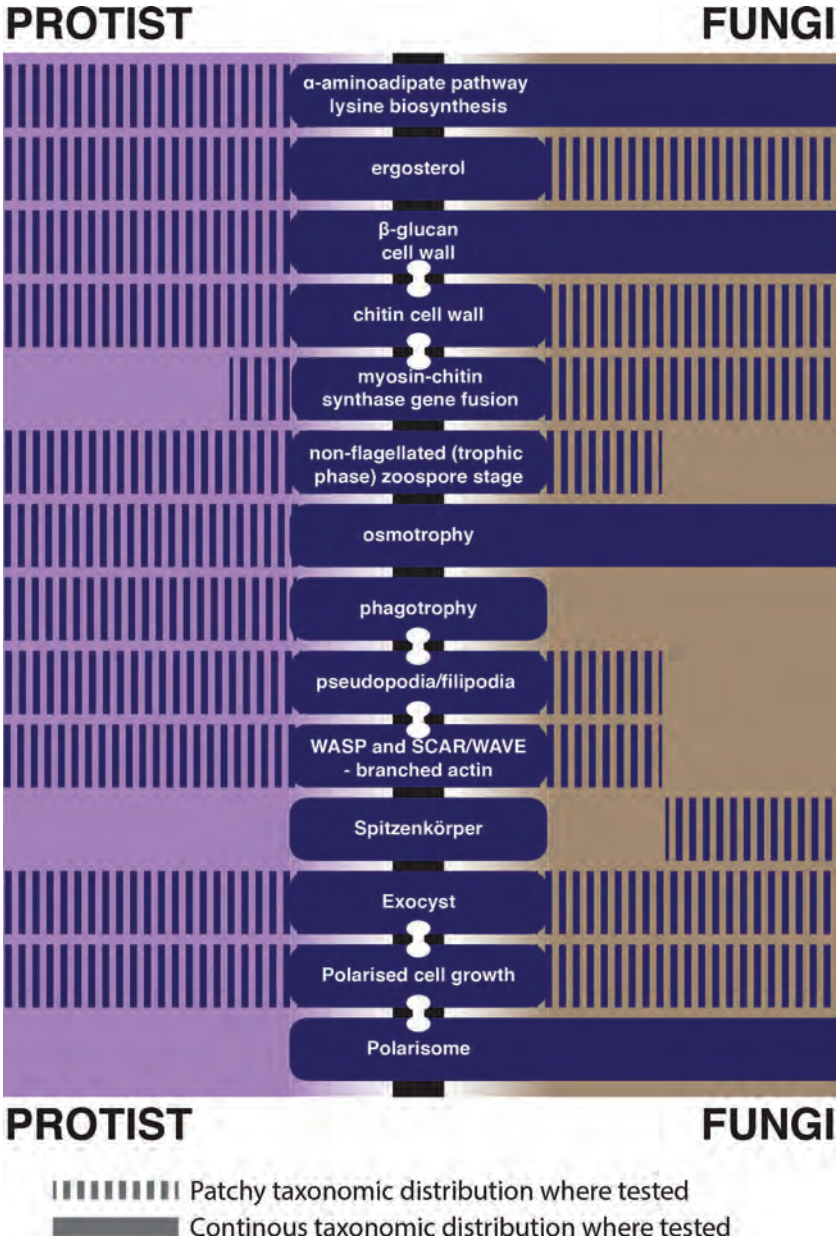


Figure 3 Cartoon illustration summarizing how features previously discussed as defining the protist-fungal transition have been shown to have a mosaic distribution within the Fungi and/or outside the Fungi among other eukaryotes. White connecting nodes illustrate linked characters/traits.

because loss represents a secondary modification. Yet the demonstration that a trait is not exclusive to the group it is said to define, and instead is present throughout the tree of life, is a knockout blow. This means that such characters cannot be used as a synapomorphy for the evolutionary group intended, unless a unique aspect of the trait can be identified which is specific to the group it is claimed to define. Likewise, we find the use of trait loss, such as the loss of phagotrophy, as a defining synapomorphy problematic because the loss of such traits has occurred on numerous occasions, which makes it difficult to see how loss can uniquely identify a group. This is further complicated by the observation that multiple chytrid lineages build pseudopodia/filipodia. Pseudopodia/filopodia are cellular systems that are prerequisites for phagotrophy, suggesting that the molecular characteristics that define the trait "phagotrophy" were not cleanly lost prior to the diversification of taxa currently unambiguously defined as fungi.

This debate is further complicated by the large number of highly diverse phylogenetic branches which are very difficult to place with or within the Fungi (summarized in Fig. 4). This confuses our understanding of character evolution. It remains a point of contention (e.g., 92, 161, 162) whether many of these groups and the associated uncultured environmentally detected diversity should be classified as fungi (such definitions are of course dependent on what characters are used to define fungi).

We do not deny that there is a unit of biodiversity that can be classified as fungi, but we argue that the line between fungi and protists is, at this point, a hostage to fortune and therefore will change given further sampling of taxa, genomes, and traits. As such, we prefer to see the transition from protist to fungi as gradual, with no clear demarcation. It therefore largely becomes a matter of emphasis where one draws the line which marks the border of the kingdom. We do not see this as a negative statement. Nor should this point be confused with philosophical arguments that can be summarized as a disagreement between the "lumpers" and the "splitters." Rather, we hope to encourage the field

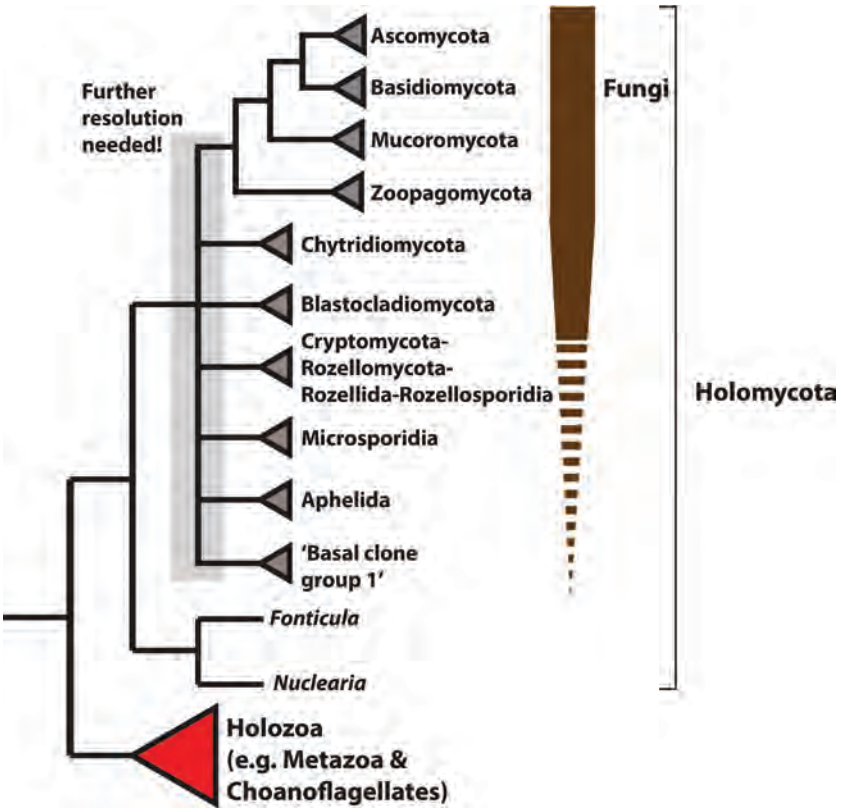


Figure 4 Schematic phylogenetic tree illustrating additional groups branching proximate to the origin of the fungal clade and the phylogenetic uncertainty among the deep branches of the Fungi and associated groups. Basal clone group 1 is composed of environmental sequences, which in some analyses is placed close to fungi (149).

to focus less on constructing categories unlikely to stand the test of time and focus more on seeking explanations for major transitions in the clade. This being said, it is perfectly possible that, with the growth of comparative data sets, a clear line can be established that separates protists and fungi. However, it would not surprise us, given what we are learning about groups historically classified as chytrids (e.g., 182), if many of these groups end up resembling protists more than fungi, which would lead to much taxonomic revision if strict naming conventions are to be imposed. This leaves us in a confusing state of affairs because we currently lack a consensus on the definition and composition of the kingdom Fungi such that it could be argued that the definition of fungi (or protist) does not matter at all. However, on an optimistic note, the investigation of the boundary between fungi and protists is going to tell us a lot about what drove the success of this evolutionarily diverse group, and this understanding, we believe, is the real prize.

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Fungal Diversity Revisited: 2.2 to 3.8 Million Species

4

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BACKGROUND

In 1825, Elias Magnus Fries (1794–1878) predicted that the fungi would prove to be the largest group in the vegetable kingdom, analogous to the insects in the animal kingdom. Notwithstanding that fungi are not actually part of the plant kingdom, how right he has proved to be as the bicentenary of his prediction approaches. By the 1960s a few mycologists were speculating that there might be as many fungal as plant species, but almost no attempts to calculate estimates from the available data were made. As concern over the conservation of biodiversity in general grew in the subsequent decades, culminating in the signing of the Convention on Biological Diversity in 1992, more precise figures on species numbers of all kinds of organisms were required. A series of estimates of the number of fungi settled on figures ranging from 500,000 to almost 10 million species, with 1.5 to perhaps 5 million receiving most support among mycologists. A recent study even predicts up to a trillion species of microorganisms globally (1); how many of these are supposed to be fungi is not specified, but if this estimate holds true and only 1% of these were fungi, the global estimate of fungal diversity would be a thousand times higher than the current highest estimate of 10 million species.

Different extrapolation techniques have been used to arrive at global fungal species richness estimates, including publication rates of new taxa (2), plant:fungus ratios (3, 4) similar to plant:insect ratios first used in Erwin's famous study (5), quantitative macroecological grid-based approaches (6–8), methods based on environmental sequence data including plant:fungus ratios (9, 10), and ecological scaling laws (1).

This article provides updated background information on the number of described species and explores new evidence obtained from rates of species discovery, extrapolations from fungus:plant ratios, and molecular sequence data from environmental samples. We conclude by indicating where undescribed species are likely to be found and recommend a working number suitable for general use in estimates of global and regional species richness.

We focus here on the numbers of species in the kingdom *Fungi*, that is, inclusive of the *Cryptomycota* and *Microsporidia*, and also including lichen-forming fungi. We do not, however, consider other organisms that mycologists study as fungi, such as the fungal analogues *Mycetozoa* (as an unranked supergroup or in the kingdom *Protozoa*, phylum *Amoebozoa*) and *Oomycota* and *Hyphochytriomycota* (in the kingdom *Heterokonta* = *Straminipila*). However, we caution that some data sets and papers discussed in the following sections are based on fungi in the broad sense and thus include information from the analogue phyla. While readers should bear this in mind, the numbers of known taxa in these groups are relatively modest, so their occasional unavoidable inclusion will not materially affect the interpretations presented here.

EVIDENCE FROM PUBLICATION RATES OF NEW TAXA

Numbers of Described Species

The number of new species described in each decade, as recorded in the Index Fungorum database (http://www.indexfungorum.org), shows three distinct phases (Fig. 1): an ascending phase in which progress was fast in the 1750s to 1860s, a steep phase as microscopy came into general use and there was intensive collecting

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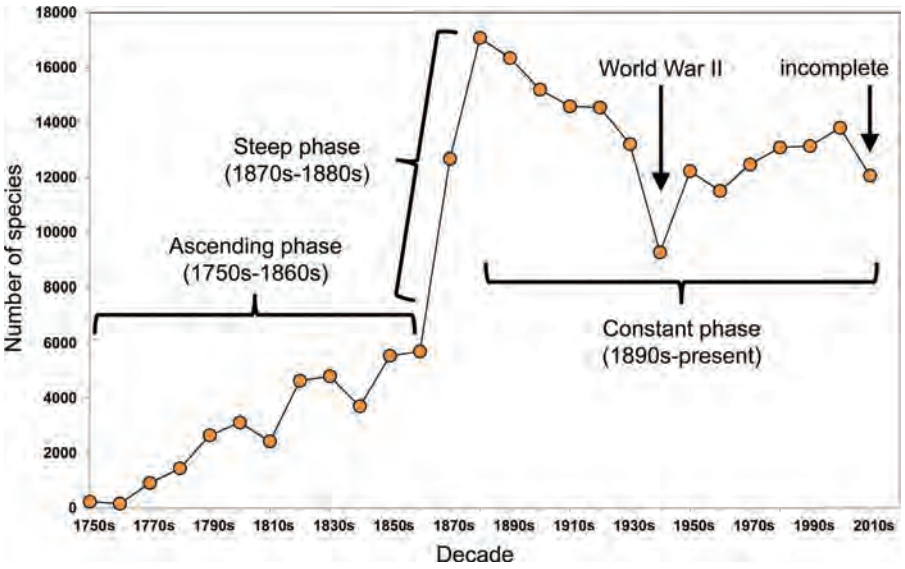


Figure 1 Numbers of newly introduced species names of fungi for each decade from 1750 to 2010. Based on data from the Index Fungorum database provided by P. M. Kirk.

in hitherto barely explored parts of the world in the 1870s to 1880s, and then a relatively constant phase from the 1890s to the present day. The partially higher figures in the first decades of the "constant phase" reflect continuing exploration and a number of prolific individual mycologists. The rate of description over the past 40 years has, however, remained fairly constant at around 1,300 per year (Fig. 2), with peaks generally relating to particular major monographic works.

Of special note is the somewhat steep increase in the rate since 2010 to around 1,800 per year. This resurgence in species description is largely attributed to the increasing use of molecular techniques for species delimitation and is of particular note because this increase followed, and even accelerated after, the ending of the separate naming of morphs of the same species in 2011 (when the provision for a Latin diagnosis or description was also eliminated). Prior to that date, the totals for

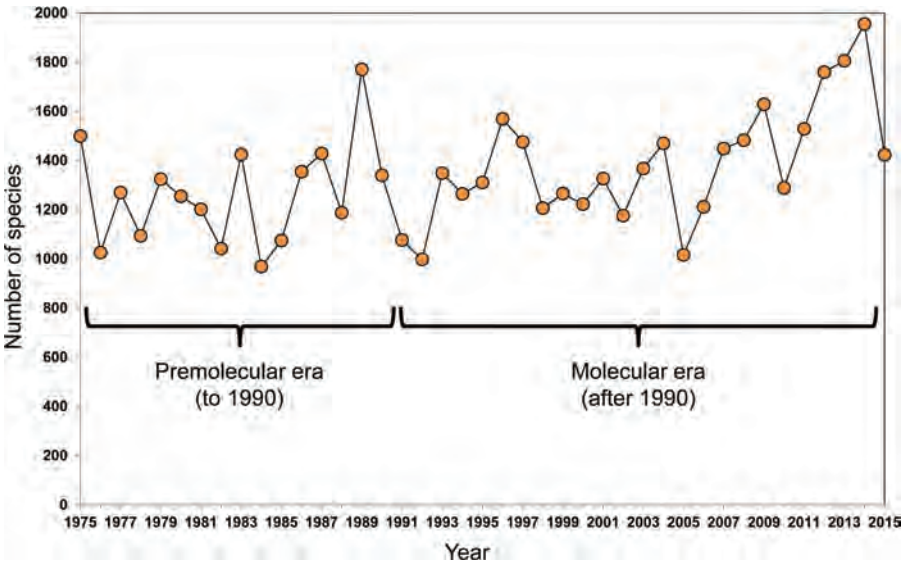


Figure 2 Numbers of newly introduced species names of fungi for each year from 1975 to 2015. Note that the data for 2015 were incomplete when this work went to press. Based on data from the Index Fungorum database provided by P. M. Kirk.

annually described "species" included separate names given to different morphs of the same species.

The number of newly named species does not, however, equal the number that have been truly described for the first time. It is much easier to name a fungus as new to science rather than to ascertain that it has previously been named. This is not simply a matter of comparing material to that of previously recognized species in the same genus but requires meticulous detective work, because not uncommonly, species were described in genera they do not belong to. Further, species concepts change, for example, in genera where morphologically identical fungi were regarded as different species if they occurred on different plant species. The number of accepted fungi can only be obtained by counting those recognized as "good" in each genus. This has been done since 1943 through the 10 editions of *Ainsworth & Bisby's Dictionary of the Fungi* and now annually through Species Fungorum inputs to the Catalogue of Life (http://www.catalogueoflife.org/annual-checklist/). An analysis of these data shows that the total number of existing species names runs at around 2 to 3 times that of currently accepted species (Fig. 3), a figure consistent with the ratio of 2.6 derived from an analysis of names in monographic works (11). The number of described good fungal species currently stands at around 120,000.

Unlike the situation in many other groups of organisms (12), the number of good fungal species continues to rise steeply decade by decade, with no indication of leveling off. The steepness of the curve would be even greater if there were more mycologists actively involved in species discovery, using either morphological or molecular approaches.

The number of species being recognized is necessarily impacted by the species concepts used. While in the premolecular era, species circumscriptions were based almost entirely on morphological and sometimes biochemical and cultural features, the incorporation of molecular information has led to a refining of species concepts. There is, however, a plethora of species concepts in biology (13, 14) and no single objective criterion that can be applied universally. Because the purpose of names is to provide a means of communication, a pragmatic species concept is essential, which may be defined as the following: "a species is what it is useful to give a species name to." (15). There have been numerous attempts to apply mechanistic systems for species recognition in phylogenetic trees, but the consolidated species concept has much to commend it and ultimately provides a broad consensus on how to recognize fungal species. The consolidated species concept adopts a polyphasic approach combining morphological, ecological, and phylogenetic species concepts (16) and is being increasingly taken up by mycologists. While in some situations this may lead to more species being recognized than otherwise would have been the case, in others it results in formerly separately distinguished

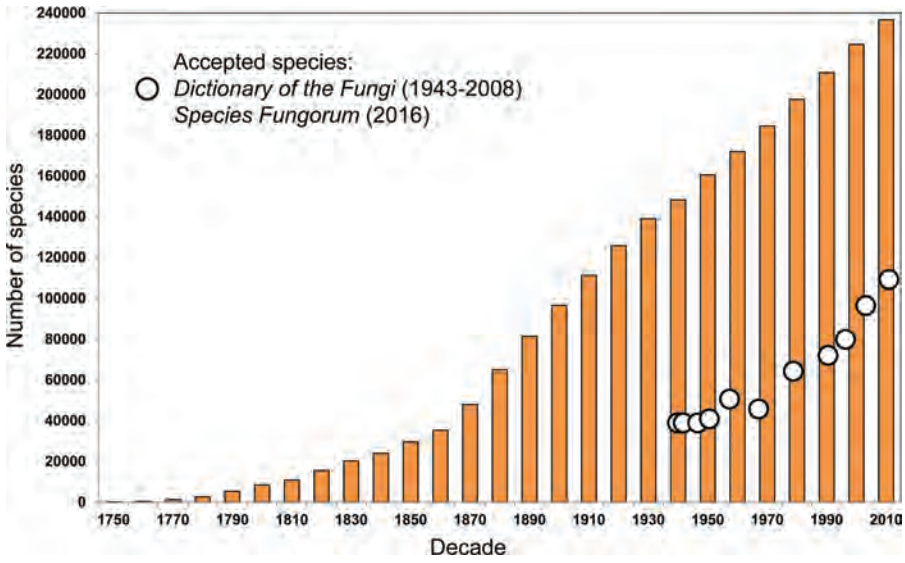


Figure 3 Growth in the total catalogued number of species names of fungi by decade from 1750 compared with the global number of accepted species. Based on figures adopted in the 10 editions of *Ainsworth & Bisby's Dictionary of the Fungi* for 1943–2008 and data in the Index Fungorum and Species Fungorum (Catalogue of Life) databases provided by P. M. Kirk.

species being united. Therefore, over- and underestimations in particular cases are usually balanced out. A rarely considered notion is the time scale: a proportion of "species" is in active speciation, with incomplete lineage sorting, yielding it difficult to resolve good species (17); in such cases, determining the number of species is philosophical, since that number is in active flux. A large number of new fungal species continue to be described with no molecular data, either due to the age of the underlying material (e.g., unveiled in older collections) or by taxonomists who do not have access to molecular techniques; assessing the proportion of good species in such taxa is therefore difficult.

Patterns in Taxon Discovery

Some predictable patterns in the recognition of published taxa in the rank of genus and above have been recognized and applied to a broad set of organisms, including fungi (2). By combining the full classifications of around 1.2 million species of all groups of organisms, these authors found, as might have been expected, that accumulation curves of higher taxa over time showed that these were more completely described and in many cases approached an asymptote. The use of the higher taxon approach was then validated by comparing the actual number of known species with that predicted from the accumulation curves. The authors then fitted asymptotic regression models to the actual curves for different groups to provide estimates of undiscovered species numbers. In the case of fungi, this led to a total number of expected fungal species of 611,000 (standard error ± 297,000). This conclusion was, however, misleading, because the analysis was based on the assumption that there were only 43,271 catalogued fungal species, rather than the nearly 100,000 accepted at that time (18). Intriguingly, a recalculation using their method with the latter species number generates a figure of around 1,400,000—not far out of line with estimates derived from extrapolations based on plant:fungus ratios (see below).

EVIDENCE FROM SPECIES RECOGNITION STUDIES

Unknown fungi are now considered to come mostly from two sources: (i) newly discovered species by means of traditional inventory methods in little-studied areas and habitats and (ii) newly discovered lineages through environmental sequencing. A further, neglected but significant source of unrecognized fungal diversity is the restudy of known taxa, applying species recognition techniques to molecular data, using either the fungal barcoding locus ITS (19) or multilocus approaches. Exemplar taxa are the fly agaric, *Amanita muscaria*, and the golden chanterelle, *Cantharellus cibarius*, which contain numerous, often regionally distributed species (20, 21). The record is currently set by the basidiolichen fungus *Cora glabrata*, which has been shown to contain at least 189 species (6, 22). We surveyed 45 such studies published between 1998 and 2016, including studies of non-lichenized and lichenized forms, and computed the ratio of species recognized in the target group before and after the study (Table 1). Most studies started out with a single or few species, whereas two looked at complexes of up to 18 species—*Protoparmelia* (23) and *Usnea* (24)—and one revised a complex of 71 species accepted prior to the study, in the genus *Coprinellus* (25). The posterior:prior ratios in the number of species recognized ranged from 0.67 (from 18 to 12 species in a group of *Usnea* [24]) to 189 (from 1 to 189 species in the basidiolichen *Cora glabrata*) (6, 22). Other high ratios were found for *Dictyonema* with 59 (26), *Cladosporium cladosporioides* with 22 (27), *Sticta weigelii* with 15 (28), *Microbotryum violaceum* with 11 (29), and *Fusarium graminearum* with 7 (30). Environmental sequence and genomic data revealed high ratios for the genera *Archaeorhizomyces*, with 145 (31), and *Cyphobasidium* with 26 (32), as well as for the still barely explored *Cryptomycota*, with 138 (33, 34), in which the true figure could be much higher. The median ratio over all evaluated studies was 3.0, and the arithmetic mean was 14.5 (Table 1).

To obtain a more reliable picture, we adjusted these ratios by geographic area covered. If a species complex had a global distribution prior to the study, and if based on material from a single geographic area, it was found to represent several species with presumably restricted distribution, the detected ratio was adjusted by the factor 2 (expanding to both hemispheres in cases in which one hemisphere was covered), the factor 3 (for tropical fungi for which a single tropical region was covered), and up to the factor 6 (for cosmopolitan fungi for which a single region within a single continent was studied). These factors were derived from selected global studies, for example, in the genera *Cladosporium* (27), *Cora* (6, 22), *Fusarium* (30), and *Sticta* (28). In some cases, we added unpublished data to the adjusted values; thus, for *Archaeorhizomyces* we currently recognize at least 500 species (B. E. Smith and R. Lücking, unpublished data), and for *Cyphobasidium*, we recognize about 100 species from deposited GenBank sequences (32). The thus adjusted ratios ranged from 0.7 (*Usnea*) to 435 (*Archaeorhizomyces*), with a median value of 10.5 and an arithmetic mean of 39.3.

To predict the global species richness of *Fungi* using these raw and adjusted ratios, we employed two further adjustments. First, we weighted the ratios according to the number of species recognized in a genus in the 2008 *Dictionary of the Fungi* (18). For instance, a genus in which a single species had been recognized received a low weight (1.0), since even a high number of newly recognized species will only contribute minimally to global fungal species richness. This adjustment avoided a high impact of extraordinary ratios such as in the previously monotypic genus *Cora*. In contrast, for groups in which a large number of species had already been recognized, the ratios based on a study of one or a few species were upweighted, e.g., in the genus *Puccinia* (35), with 4,000 accepted species. In addition, to account for ecological bias in the evaluated studies, we applied a group weight according to the major lifestyle attributed to each genus; to that end, we divided the approximately 100,000 known fungi in 2008 as follows: 40,000 saprotrophic (weight 4.0), 20,000 phytopathogenic (weight 2.0), 20,000 lichenized (weight 2.0), 8,000 ectomycorrhizal (weight 0.8), 5,000 endophytic (weight 0.5), 3,000 entomopathogenic (weight 0.2), 2,000 aquatic (weight 0.2), 500 endomycorrhizal (weight 0.05), 500 marine (weight 0.05), 400 soil (weight 0.04), and 100 human pathogenic (weight 0.01). The categories aquatic, marine, and soil are used here for cases where the habitat is known but not the biology. These proportions denote estimates based on known fungi in 2008, not estimated proportions of extrapolated global species richness, in which, presumably, categories such as plant pathogens and soil fungi will have much higher values. Using these weights, the overall arithmetic mean of the posterior:prior ratio for all evaluated studies amounted to 11.3. This suggests that even without including important sources of new species discoveries, the already accepted *Fungi* may contain up to 10 times as many species as currently recognized, resulting in more than 1 million estimated species. Remarkably, this is about 4 times higher than the currently estimated synonymy ratio of 2.6, suggesting that a large proportion of these unrecognized species have no names available.

EVIDENCE FROM EXTRAPOLATIONS BASED ON PLANT:FUNGUS RATIOS

The widely cited and accepted plant:fungus ratio-derived global estimate of 1.5 million species of fungi was based on information from several independent sources (3): (i) the numbers of fungi reported from all habitats in the United Kingdom and sites within the United Kingdom compared with the number of plant species present, (ii) the numbers of host-specific fungi on particular plant species, (iii) percentages of new species being discovered, and (iv) extrapolations to the global level based on conservative estimates for the number of plant species, making allowances to discount separately named morphs of the same species. The 1.5 million figure was considered conservative because, while numbers of fungi from all sources, including organisms other than plants and soil, were included in the species totals for particular places, no allowances were made for undiscovered fungi in and on the millions of insects predicted to exist.

Some figures used in these early extrapolations have subsequently been revised upward. Ten years later, it was pointed out that the number of plant species and the fungus:plant ratio were too conservative and the vast number of insect fungi had not been considered. As a consequence, some revised estimates ranged between 2.7 and 9.9 million fungal species (4).

The total inventory of fungal species found in field surveys in a particular site continues to increase year by year, even with regular visits spanning several decades, while the number of plant species remains more or less unchanged. Esher Common (Surrey, United Kingdom) is by far the most investigated site by field mycologists in the world, and to date about 3,400 species have been found (B. M. Spooner, personal communication). Because the number of vascular plant species remains at 420, this now gives a fungus:plant ratio of 8:1.

Some preliminary results from next-generation sequencing of soil samples from a wood within the second most field-surveyed site in the world, the Slapton Ley National Nature Reserve in southwest England, are illuminating in relation to the fungus:plant ratios (G. W. Griffith, B. S. Dentinger, and D. L. Hawksworth, unpublished). The wood studied has 1,136 species of fungi and 88 vascular plant species recorded during field surveys, a ratio of 12.9:1. However, just 686 sequence-based species (accounting only for taxa represented by five or more sequences) were recovered from soil samples (and a further 153 taxa were represented by one to four sequences). What is intriguing is that of the 164 named genera represented in the next-generation sequencing data set, only 32 were among the 549 genera collected in the fieldwork, which included fungi on plant parts and plant litter, not just sporophores emerging from the soil. The actual ratio in a site may therefore be substantially higher than indicated by traditional inventory techniques.

The interpretation of some data sets needs to be considered in the light of such long-term investigations. Ratios comparing fungal sequences recovered

Table 1 Selected species recognition studies in different groups of fungi

Taxon	Genus[a]	Lifestyle	Coverage[b]	Range[c]	Ratio[d]	Factor[e]	Adjusted[f]	Weight[g]	Reference
Acantholichen pannarioides	1	Lichenized	Neotropical	Neotropical	6	1	6	8	100
Amanita muscaria	500	Ectomycorrhizal	N. Hemisphere	Global	6	2	12	6,000	20
Archaeorhizomycetes	0	Soil fungus	N. Hemisphere	Global	145	3	500	500	31
Aspergillus flavus	266	Saprotrophic	Australia	Global	2	6	12	3,192	101
Aspergillus fumigatus	266	Human pathogenic	Global	Global	2	1	2	532	102
Auxarthron zuffianum	15	Saprotrophic	Global	Global	2	1	2	30	103
Beauveria	9	Entomopathogenic	Global	Global	4	1	4	36	104
Blastomyces dermatitidis	1	Human pathogenic	N. America	Global	2	1	2	2	105
Botrytis cinerea	54	Phytopathogenic	Europe	Global	2	6	12	648	106
Calonectria pauciramosa	34	Phytopathogenic	Global	Global	3	2	6	204	107
Cenococcum geophilum	1	Ectomycorrhizal	N. America	Global	5	6	30	30	108
Cladosporium cladosporioides	150	Saprotrophic	Global	Global	22	1	22	3,300	27
Coccidioides immitis	3	Human pathogenic	N. America	N. America	2	1	2	6	103
Coniophora puteana	20	Saprotrophic	Global	Global	3	1	3	60	109
Coprinellus	100	Saprotrophic	Global	Global	1,2	1	1.2	120	25
Cora	1	Lichenized	Subglobal	Global	189	1	189	189	6, 22
Corella	0	Lichenized	Central/S. America	Central/S. America	11	1	11	11	26
Cryptomycota	0	Aquatic	N. America	Global	39	6	234	234	33
Cyphellostereum	1	Lichenized	Central/S. America	Global	15	3	45	45	26
Cyphobasidium	0	Lichenicolous	N. America	Global	26	4	100	100	32
Dictyonema	6	Lichenized	Subglobal	Global	59	3	177	1062	26
Fusarium graminearum	111	Phytopathogenic	Global	Global	7	1	7	777	30
Grosmannia clavigera	1	Phytopathogenic	N. America	N. America	2	1	2	2	110
Hymenoscyphus albidus	155	Phytopathogenic	Europe	Europe	2	1	2	310	111
Lasiodiplodia theobromae	9	Phytopathogenic	Pantropical	Pantropical	3	1	3	27	112

Letharia	2	Lichenized	N. Hemisphere	N. Hemisphere	3	1	3	6	113
Lobariella	5	Lichenized	Neotropical	Neotropical	3.8	1	3.8	19	114
Metarhizium anisopliae	9	Entomopathogenic	N. America	Global	2	6	12	108	115
Microbotryum violaceum	87	Phytopathogenic	Europe/N. America	Global	11	4	44	3,828	29
Neofusicoccum parvum/ribis	13	Phytopathogenic	Regional/host	Global	2	60	150	1,950	116
Paracoccidioides brasiliensis	1	Human pathogenic	S. America	S. America	4.0	1	4	4	117
Parmelia	95	Lichenized	Europe/N. America	N. Hemisphere	3	1.5	4.5	428	118
Parmelia saxatilis	1	Lichenized	N. America	Global	4	3	12	12	119
Parmotrema reticulatum	348	Lichenized	Subglobal	Subglobal	4	1	4	1,392	120
Phialocephala fortinii	1	Endophytic	Europe	Global	7	6	42	42	121
Protoparmelia	20	Lichenized	Global	Global	1.6	1	1.6	32	23
Puccinia monoica	4,000	Phytopathogenic	N. America	Global	1.8	6	10.5	42,000	35
Rhizoplaca melanophthalma	11	Lichenized	N. America	Global	2.5	6	15	165	122
Stachybotrys chartarum	44	Saprotrophic	N. America	Global	2	1	2	88	123
Sticta fuliginosa	114	Lichenized	Global	Global	15	1	45	5,130	28
Sticta weigelii	114	Lichenized	Global	Global	23	1	23	2,622	28
Strobilomyces	20	Ectomycorrhizal	Asia	Global	3.5	6	21	420	124, 125
Tricholoma scalpturatum	200	Ectomycorrhizal	Europe	Global	2	6	12	2,400	126
Uncinocarpus reesii	3	Saprotrophic	Global	Global	2	1	2	6	103
Usnea	338	Lichenized	N. Hemisphere	N. Hemisphere	0.7	1	1	338	24

[a]Number of species recognized in the corresponding genus in reference 18.
[b]Origin of material used in the study.
[c]Global distribution of the group.
[d]Ratio of the number of species recognized after versus before the study.
[e]Geographic adjustment.
[f]Adjusted ratio based on raw ratio and geographic adjustment.
[g]Weight based on adjusted ratio and original number of species recognized in the corresponding genus in reference 18 (in some instances [*Acantholichen*, *Sticta*, *Usnea*] further adjusted based on unpublished data).

from environmental samples with plants growing on the sample sites have given some surprising ratios. For example, data from a forest in North Carolina gave fungus:plant ratios of 19:1 (491 fungi and 26 plants) and 13:1 (616 fungi and 48 plants), leading to a suggestion that there may be 3.5 to 5.1 million fungal species worldwide (9). A more modest ratio of 7.5:1 (1,500 fungi and <200 plants) was obtained from boreal forest soils in Alaska (36). In contrast, Tedersoo et al.'s (37) impressive study of 365 globally distributed soil samples using metabarcoding molecular methods (see below) led the authors to conclude that fungal species richness had actually been overestimated by 1.5 to 2.5 times from that based on plant:fungus species ratios. Any such extrapolation from soil samples alone to the total fungal biota at a site is, however, unsound as evidenced by the above data from the Slapton reserve. Much of the actual fungal species diversity in a site will inevitably be missed in soil sampling, because most is above ground, on and in plants and animals of all kinds, in water, in lichens, on rocks, etc.

Collections made in brief smash-and-grab collecting trips or even extending over several years will only start to reveal what fungi may actually be present at a site. Studies such as that of Piepenbring and collaborators (38), in which 567 species of fungi and 311 species of plants were found along a 500-m pathway in Panama over 2 years, giving a ratio of just 1.8:1, have to be interpreted in this context. The ratio resulting from that study would be expected to rise significantly if the period over which it was conducted had been 2 decades rather than 2 years, and with increased attention to microscopic fungi.

When considering fungi of all biologies and in all ecological niches in a site, the ratio can be expected to vary depending on the geographical location, as recognized some 25 years ago (3). It is not surprising, therefore, that the studies of fungus:plant ratios in a site, obtained by field survey and molecular approaches, have generated ranges from 1.8 to 19.1:1. The mean of the figures cited in this section yields a ratio of 9.8:1, suggesting that the ratio of around 6:1 arrived at in 1991 may be conservative. With the currently accepted number of approximately 380,000 vascular plant species (39), this adjusted ratio would predict a number of nearly 3.8 million fungal species.

EVIDENCE FROM ENVIRONMENTAL SEQUENCING TECHNIQUES

The advent of obtaining taxon-specific, molecular sequence data from environmental samples, most commonly soils but also water and plant tissues, has generated a new source of data for estimating global species numbers. It is commonly found that numerous sequences thus obtained do not have any matches with those from named fungi in GenBank (see below), and this has led to speculations that previous estimates have been too low.

Generalizing data from environmental samples is problematic for several reasons, including that the broader geographic context of detected operational taxonomic units (OTUs) is usually unknown. For example, using 454-sequencing technology, two 0.25-g soil samples from an Alaskan forest collected just 1 m apart had only 14% of fungal species in common (36). In a pyrosequencing study in the Andes, 1,839 species-like taxa were found, of which 25% were most similar to other unidentified environmental samples, with significant differences between forests at different altitudes (40). The most comprehensive study of soil samples to date is that of Tedersoo et al. (37), who analyzed 2-g soil samples from pooled soil in 5-cm deep cores taken from 365 global sites. They obtained 80,486 fungal OTUs, used a 98% sequence similarity for species recognition, and did not consider almost half in further analyses because they were singletons and potentially artifacts; in that study, plant:fungus richness declined toward the poles. However, in concluding that extrapolations from plant:fungus ratios were unjustified (see above), the authors did not take into account fungi other than those in soil, such as those in and on aerial parts of plants, in decaying vegetation, lichen-fungi, and entomogenous fungi.

Especially instructive is a study of 928 swabs of dust samples across the continental United States by direct PCR and using high-throughput sequencing (HTS) to analyze them (41); not only were 38,473 fungal taxa detected, but there were clear geographic patterns with a predictive value of placing a sample within 230 km. Of the 38,473 taxa, however, sequences of about 40% could not be matched with any named species (R. R. Dunn, personal communication).

Just how many unidentified sequences generated from environmental samples represent undescribed species is uncertain. Compared with the 120,000 formally recognized species (see above), as of 25 November 2016, there were only 34,878 named fungal species in GenBank, but there were a further 94,059 species-level OTUs with no names (C. L. Schoch, personal communication); these figures have increased by 68% and a staggering 117%, respectively, in the past 5 years. However, only a minute fraction of environmental sequences are deposited individually in GenBank; the vast

majority is placed in the Sequence Read Archive (SRA) (42). Currently, the SRA, on the query "(fungal OR Fungi) AND (internal transcribed spacer [or, ITS] OR ITS1 OR ITS2)," returns 179 studies, 1,822 biosamples (i.e., environmental samples), and 14,334 experiments or HTS runs, containing 928 million fungal ITS reads, with an average length of 353 bases (SRA: https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=search_obj; accessed 12 March 2017). In contrast, using the same query, GenBank (43) returns 993,987 sequences obtained predominantly through Sanger sequencing (GenBank: https://www.ncbi.nlm.nih.gov/genbank; accessed 12 March 2017). Thus, at present there are almost 1,000 times more HTS reads than Sanger sequences. Only 3 years ago, this ratio was 18:1, which means it has increased by a factor of more than 50 in this short period.

Obviously, a proportion of the unidentified sequences can be expected to represent known but as yet unsequenced species, especially considering that about 85,000 of the currently accepted fungal species have no sequence data available. In the case of *Mortierella*, a reference set of type and other strains was sequenced and then compared with unnamed sequences in GenBank (44); the authors modeled the effects of increasing type strain sequencing and found a linear relationship with the number of strains that could be identified, and they predicted a number close to that of the already described species.

One of the most significant problems with using environmental sequence data to extrapolate global fungal species richness is the approach to define OTUs. Due to the volume of data generated and the usual broad taxonomic range detected, alignment-based methods that would allow us to critically define species as phylogenetic lineages are impossible to use as routine techniques. Thus, environmental sequence data are analyzed using clustering techniques, and OTUs as equivalents to species are defined on thresholds, ranging between 95 and 99% depending on the study. This approach has several shortcomings. First, species do not exhibit fixed sequence thresholds; in contrast, sequence divergence and hence the barcoding gap depend on the evolutionary age of the species complex. Second, commonly used thresholds of 95 to 97% are unrealistic. Assuming that the ITS barcoding locus has a length of, e.g., 500 to 600 bases, 5% divergence would correspond to 25 to 30 bases. In most studied species, infraspecific ITS variation is much lower, with an average of about 1%. Theoretically, a more realistic threshold of 99% would greatly increase the number of OTUs detected in a data set. However, this effect is more than outweighed by the sensitivity of clustering methods to variation in the data, including the common CAFIE (carry-forward-incomplete-extension) sequencing error common to all base flow technologies (45) (see below).

This error and other problems with clustering techniques can lead to an overestimation of the number of OTUs at several orders of magnitude, which may result in serious bias when using such estimates for global extrapolations. We tested this effect with SRA data of the soil fungus *Archaeorhizomyces* (Smith and Lücking, unpublished). Through a blast script, we obtained 106,563 ITS reads from the SRA that were reasonably close to the type species, *Archaeorhizomyces finlayi*. Clustering analysis using USEARCH (46) resulted in the following numbers of OTUs depending on the set threshold: 2,658 at 95%, 5,793 at 97%, 10,630 at 98%, and 28,435 at 99%. Thus, the difference between the 95% and the 99% threshold is an order of magnitude. Alignment-based phylogenetic analysis suggests the presence of approximately 500 species-level lineages in this data set. Thus, depending on the threshold level used for species-level separations, clustering could overestimate the actual species richness in this case by a factor of between 5 and 50.

Lücking and colleagues (45) showed that severe problems with estimating OTUs through clustering emerge from even minor sequencing errors. The CAFIE error is common to all next-generation sequencing technologies, because it represents a statistical error of the distribution of bases among wells on a plate through a given base flow. Through careful adjustment, this error can be held to a minimum of less than one erroneously phased insertion per read, i.e., about 0.3% assuming an ITS1 or ITS2 read length of approximately 300 bases. This proportion is well within the expected infraspecific variation even at a high level of 99% similarity and thus theoretically should disappear as background signal. However, the random distribution of this error across the entire sequence, which cannot be recognized by quality filtering methods, especially not in environmental samples where no expected sequence patterns exist for comparison, causes clustering methods to interpret these differences as taxonomic, leading to serious overestimations by distribution sequences of the same taxon, but with different minor errors, into separate clusters. This is because clustering methods work with local alignments of small, highly similar sequences, whereas phylogenetic methods require alignment of sequences of a data set to template sequences obtained from Sanger sequencing. As a consequence, in a broad alignment including Sanger reference sequences, erroneous and phased indels will be located

in mostly gapped columns, which have practically no influence on the resulting topology, whereas in clustering methods, they will frequently be aligned with nonhomologous base calls and falsely interpreted as substitutions (45).

In the above (45), a proportion of less than 1% erroneously phased indels caused by CAFIE errors in the ITS of a single species caused overestimation of the number of taxa by a factor of 35 at a 95% threshold level, 137 at 97%, and 980 at 99%. Thus, instead of disappearing as background signal, the sequencing error had a tremendous effect on OTU evaluation when in that case it was known that all sequence data came from a single target species. Even after removing all erroneous indels, clustering continued to overestimate species richness by a factor of 4 at a 95% threshold level, 8 at 97%, and 116 at 99%, due to minor remaining infragenomic variation and other sequencing errors. In contrast, alignment-based phylogenetic analysis of a broad set of nearly 2,000 reads recovered a single species, even with all sequence errors included. Thus, while environmental sequencing continues to accumulate a vast amount of important data on unrecognized fungi, extrapolations from these data must be taken with great care when based on clustering methods. An extreme example is a recent study which predicts up to a trillion species of microorganisms globally based on OTUs derived from environmental sequence data (1), using ecological scaling laws which, put in simple terms, predict how the global, limited number of *N* individuals would divide into *S* species, taking into account resource competition based on body size and other factors. This number, while certainly intriguing, appears to be exaggerated, and it would be interesting to see what alternative prediction would be obtained when defining the underlying taxonomic entities in this analysis through alignment-based phylogenetic methods.

In spite of these shortcomings, it is clear that environmental sequencing is now the major source of discovering novel fungal taxa, at least in the same order of magnitude as taxa recovered in species recognition studies or perhaps much higher. It follows that to properly catalog these fungi, a naming system for voucherless sequences must be developed, and proposals on how to do that are currently under consideration (47–51).

In addition to discovering new species, environmental sequencing may reveal unknown higher fungal taxa up to the class level. The best-known example is the recently discovered class *Archaeorhizomycetes* (31, 52). In Tedersoo and colleagues' study (37), ~6% of all fungal OTUs below the phylum level could not be assigned to any known class. Further clustering of unidentified fungal sequences at 70% sequence similarity revealed 14 distinct taxonomic groups comprising >7 OTUs, suggesting that there are several deeply divergent class-level fungal lineages that have not yet been described or previously sequenced. A follow-up study by the same group (53) also revealed numerous new lineages at least at the order level.

WHERE ARE THE UNDESCRIBED FUNGI?

If there are at least 1.5 million or more fungi on Earth, and we know of just 120,000, where can the missing 1.38 million species be? As outlined, three major sources for unrecognized fungal diversity seem to emerge: (i) geographic areas and ecological habitats that are largely understudied, particularly in tropical regions and biodiversity hot spots, (ii) ecologically cryptic fungi that occur in the environment but usually do not manifest themselves via discernible structures other than microscopic hyphae and mycelia, and (iii) so-called cryptic species hidden under well-established names.

Biodiversity Hot Spots

It is generally recognized that missing species of all groups of organisms will be found in biodiversity hot spots (54). A novel quantitative method to identify hot spots of unrecognized species richness is the grid approach (6–8), which relies on linear interpolation and hence gives more reliable estimates than nonlinear extrapolation. The area in question is divided into grids, and observed richness per grid is linearly correlated with predictive macroenvironmental parameters and sampling effort, using at least one well-sampled grid for calibration. This method can be applied to both traditional inventories and data based on environmental sequencing, as long as species are reliably delimited and macroenvironmental predictors can be reasonably derived from the data. Thus far, the method has been employed for lichen-forming fungi in the families *Graphidaceae* and *Trypetheliaceae* and the genus *Cora*, resulting in estimates ranging between 1.5 and 4 times the number of known species.

South America is generally recognized as having major hot spots, and various studies point to high levels of novelty there. By combining data from basidiomes and sequence data from ectomycorrhizal roots in a *Dicymbe corymbosa* forest in Guyana, Henkel and collaborators (55) estimated that over 250 species were associated with this one tree species in this single site. López-Quintero and colleagues (56) found that of 632 macromycete species in Colombian Amazonian forests, 52%

could not be identified to the species level, a significant proportion likely representing undescribed species. Truong and coworkers (57) collected 1,430 basidiomes during just four collecting expeditions in southern South American *Nothofagaceae*-dominated forests; they generated 439 OTUs out of 957 specimens, of which 308 (ca. 70%) did not match any in the UNITE dynamic database at 97 to 99% similarity and thus did not correspond to any "species hypothesis" currently in the database.

Little-Explored Habitats

There are an enormous number of potential sites for fungi in any locality, and in the case of the tropics at least 31 ecological niches meriting study can be recognized, many requiring different techniques and specialists to explore (58). While no site on Earth can yet be considered to have a complete inventory of the fungi present, some habitats stand out as particularly underexplored.

The situation can be exemplified by the fungi which obligately grow on lichens, the lichenicolous fungi. With a few notable exceptions, this niche was largely overlooked by lichenologists and other mycologists until the mid-1970s. In 1976, there were just 457 species known worldwide (59), but by 2016 that number exceeded 1,800 (http://www.lichenicolous.net/); that represents a 394% increase over 40 years, or 98.5% per decade. This growth is reflected at the more local level, where in Great Britain and Ireland the number known rose from just 218 species in 1983 to 403 in 2003, an increase of 85% over 20 years (60); the number has now passed 500 and continues to rise (D. L. Hawksworth, unpublished). That figure compares with around 1,900 lichenized species in the same region, implying by extrapolation to the global scale that there may be as many as 25% as many lichenicolous fungi as lichenized species, i.e., around 5,000 species. This hypothesis is supported by studies of these fungi in areas where they have not previously been investigated; for example, of 189 species reported from Isla Navarino in Chile on the basis primarily of collections made in just 1 year, 6 genera and 60 species (32%) were new to science (61). And these figures do not include the so-called endolichenic fungi that can be cultured from lichen thalli or are only known from sequence data (e.g., 62, 63). In addition, emerging studies suggest the presence of highly specific, yet morphologically cryptic, basidiomycete yeasts and other basidiomycetes in the cortex of lichen thalli, which add yet another dimension to unknown fungal diversity (32, 51).

The experience with lichenicolous fungi appears or can be expected to be paralleled in other little-explored habitats, including bryophytes (64–66), algae (67, 68), endophytic fungi inside vascular plants (69–71), tropical foliicolous and fungicolous fungi (72), mammal guts (73), insect guts (74–77) and exoskeletons (78, 79), on and in rocks (80, 81), and in deep sea and ocean sediments (82), to name just a few.

Cryptic Species

Biologically distinct species which are morphologically indistinguishable, so-called cryptic species, were recognized 2 decades ago as one place where some of the predicted "missing fungi" might be discovered, since physiological and other biological divergence evidently often precedes morphological divergence in the evolution of fungal species. Based on studies of selected complexes then available, it was suggested that the number of known fungi might rise by a factor of 5 or more for this single reason (83). Molecular studies reveal that cryptic speciation is widespread through diverse groups of fungi, including plant pathogens, clinically important fungi, lichen-forming fungi, and mushrooms. Recognition of such cryptic speciation is indeed playing an increasingly important role in species discovery, as outlined above, and the ratio derived from current studies, 11.3:1, is about twice as high as estimated by Hawksworth and Rossman (83).

Existing Reference Collections

It has been estimated that over half of the projected 70,000 undiscovered plant species have already been collected and await description (84). Similarly, most reference collections of fungi, whether of dried specimens (fungaria) or living cultures (culture or microbial resource collections), include material that is not named to the species level, and in some cases not even to the genus level. Further, some collections and isolates referred to named fungi not uncommonly prove, on critical examination, to be different species. It has been suggested that around 20,000 fungal species have been collected but are as yet undescribed (83), but that is likely to be an underestimate because many mycologists have large backlogs of material awaiting formal description.

One of the challenges in the linking of newly obtained sequence data with named material in collections, particularly those that are type material, is fixing the application of the species name. Currently, the 34,878 species with sequences in GenBank (see above) represent just 29% of the 120,000 known species. Obtaining sequences for the missing 71% of named accepted species is essential to determine the novelty of recovered sequences from the environment, specimens,

or isolates. While some researchers have had considerable success in recovering DNA from dried specimens (85), even from a dried *Hygrophorus* basidiome collected as far back as 1794 (86), this is not always possible (87). Aged dried material generally exhibits high levels of DNA degradation, making access through conventional extraction and PCR methods difficult (88, 89). In view of the serendipity of obtaining satisfactory results, collection curators are often unwilling to allow parts of often scant, irreplaceable type specimens to be destroyed for DNA extraction. Several attempts are now being made at using high-throughput sequencing technologies to obtain sequence data from highly fragmented DNA (88). One of the main problems in this respect is contamination, since even for highly variable loci, it will be a challenge to piece small fragments together without the risk of generating artificial chimeras. Therefore, efforts should be made to identify highly diagnostic regions in the ITS or other loci that allow the identification of a taxon even when only short fragments are at hand (89), as exemplified by comparing the ITS1 and ITS2 subregions (90).

There is a particular problem regarding the type species of generic names. An impressive 5,317 generic names of fungi are represented in GenBank (C. L. Schoch, personal communication), but in many cases the sequences are not from the name-fixing type species. A concerted attempt has been initiated to sequence the type species of genera currently not represented in DNA databases. This involves recollection and/or isolation from the geographical area of the original material and from the same host or substrate when no DNA has been recovered from the type material (91). Sequenced material of species can then in some cases be designated as an interpretive type—an epitype (92). Such an effort involves global collaboration of sequencing laboratories with colleagues in countries where high unknown diversity is located.

TOWARD A WORKING NUMBER OF GLOBAL FUNGAL SPECIES RICHNESS

There is general acceptance among mycologists that the global number of fungal species is a seven-digit figure, in the range 1 to 5 million (10), with "at least" 1.5 million now predominating, though a possible order of magnitude higher has been hinted at (93) and a minimum figure of 611,000 to 712,000 was arrived at (2, 94), leaving aside the figure of potentially billions of fungi extrapolated by Locey and Lennon (1). A recalculation of the number computed via Mora and colleagues' approach (2) gives a figure of about 1.4 million (see above), close to the 1.5 million estimate which has come to be widely accepted by other biologists (54, 95–97), notwithstanding a few estimates that fail to appreciate the difficulty of inventorying fungi (98) or that equate numbers detected in soil alone as indicative of the total number at a site (37).

Unfortunately, the different techniques used to estimate global species richness are in part additive and in part overlapping or redundant, so that a combination of estimates from these studies is difficult. Thus, the revised estimate of nearly 3.8 million fungal species based on an updated fungus:plant ratio of 9.8:1 and 380,000 vascular plant species should include fungal species of all sorts with all types of ecologies and detected by different methodologies, including environmental sequencing and species delimitation methods. This number should then be congruent with approaches such as publication rates or scaling methods, but these approaches result in widely disparate estimates. Alternatively, an additive approach would be based on the three major sources of unrecognized fungal species richness, which can be reasonably sorted in descending order into (i) environmental sequencing, (ii) cryptic speciation, and (iii) other novel discoveries through traditional field work. For cryptic speciation, the above survey of established literature results in a weighted ratio of about an order of magnitude, i.e., more than 1 million additional species if based on 120,000 previously known species. If novel species from environmental sequencing are at least as high in number, this would add at least another million.

We conservatively assume that other novel discoveries (excluding species delimitation methods and environmental sequencing) will occur at a proportion of at best 10% of the latter two approaches. This assumption is based on the rate of 1,300 newly described species per year until about 2010, before the onset of rigorous species delimitation methods and environmental sequencing (see above). At this rate, it would take nearly 100 years to double the number of known species. Thus, while environmental sequencing and species delimitation methods would contribute about 2 million new taxa, other novel discoveries would add at best another 120,000 taxa within a reasonable time frame, for a total of a little over 2.2 million. This would give a range of between 2.2 (additive approach) and 3.8 million (global ratio approach), very much in line with the previously updated estimates of between 2.3 and 3 million (4, 99) and precisely narrowing down the range of 1 to 5.1 million given by Blackwell (10) by 1.2 to 1.3 million on either side.

We therefore propose to replace previous estimates of global fungal species richness with this updated range

of 2.2 to 3.8 million. This estimate is likely to be further improved on when reliable statistical approaches to analyze the huge amount of environmental sequence data become available. An interesting approach would be to combine the following techniques: (i) a geographically and ecologically broad sample of environmental sequence data, (ii) alignment-based species recognition methods to properly estimate OTU diversity, (iii) species-based niche modeling to establish macro- and microecological patterns, and (iv) a grid-based interpolation of global species richness, taking into account sampling effort. We predict that such an analysis will result in estimates that might lie well above the revised conservative estimate of 2.2 million species and likely even beyond 3.8 million species. But even if continuing to adopt the previous, now appearing highly conservative number of 1.5 million species, the discovery and formal description of the missing fungi remain a daunting task.

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Microsporidia: Obligate Intracellular Pathogens Within the Fungal Kingdom

5

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INTRODUCTION

Microsporidia were initially described about 150 years ago with the identification of *Nosema bombycis* as the organism responsible for the disease pébrine in silkworms (1). Microsporidia are ubiquitous in the environment and infect almost all invertebrates and vertebrates, as well as some protists (2). Spores from microsporidia are commonly found in surface water (3). These organisms are eukaryotes that have a nucleus with a nuclear envelope, an intracytoplasmic membrane system, chromosome separation on mitotic spindles, vesicular Golgi, and a mitochondrial remnant organelle lacking a genome termed a mitosome (4). For insects, fish, laboratory rodents, and rabbits microsporidia are important pathogens, and they have been investigated as biological control agents for destructive species of insects (2). Several species of microsporidia have caused significant agricultural economic losses including *Nosema apis* and *Nosema ceranae* in honeybees (5), *Loma salmonae* in salmonid fish (6), and *Thelohania* species in shrimp (7). Franzen (8) published an excellent review of the history of research on these pathogens, and a recent textbook by Weiss and Becnel (2) provides a comprehensive examination of what is known about these organisms. In 1977 Sprague elevated the class or order Microsporidia to the phylum Microspora (9), and a decade later Sprague and Becnel (10) suggested that the term Microsporidia should instead be used for the phylum name. These organisms were previously considered "primitive" protozoa (11), but molecular phylogenetic analysis has resulted in the insight that these organisms are not primitive but instead are degenerate, and that Microsporidia are related to the Fungi, either as a basal branch of the Fungi or as a sister group (12–16).

The majority of microsporidia initially infect their hosts via the gastrointestinal tract, and they have evolved an infection apparatus, the polar tube, which allows them to infect host cells at a distance, enabling them to traverse the space between the lumen of the gastrointestinal tract and the host cells lining the digestive system (2). Many of the microsporidia disseminate following initial infection, and there are descriptions of microsporidian infections in almost every organ system, with human cases of encephalitis, ocular infection, sinusitis, myositis, and disseminated infection being well documented in published literature (2, 17). Microsporidia can have significant effects on their hosts and host cells, with infection resulting in juvenilization, feminization, or other changes to host physiology, as well as the formation of xenomas or other multinucleate cellular structures (2). Recent work to investigate microsporidia-host interactions has demonstrated the complexity of this interaction and the effects of microsporidia on the host cell transcriptional response in model systems of this interaction, including insects and *Caenorhabditis elegans* (18–20).

Human infection with microsporidia causing encephalitis was initially described in 1959. The species of microsporidia described to date in human infection suggest that most infections in humans are zoonotic and/or waterborne. Microsporidiosis has been a particular problem in immune suppressed hosts, such as those with advanced human immunodeficiency virus infection (AIDS) (21) and patients on immune suppressive drugs such as patients with organ transplantation or those receiving immune modulatory antibodies such as anti-tumor necrosis factor alpha (21, 22). Microsporidian infections in immunocompetent mammals are often chronic and asymptomatic, while immunocom-

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promised hosts often develop lethal infections (23). Diarrhea due to microsporidiosis from *Enterocytozoon bieneusi* was initially reported in patients with AIDS in 1985 (24), and the number of articles describing human disease increased dramatically after 1990 with improvements in diagnostic techniques (2, 21, 25). The development of effective combination antiretroviral therapy has resulted in a decline in the cases of microsporidiosis in patients with HIV infection (2, 26). As noted above, however, infection can also occur in immunocompetent individuals, and in particular, diarrhea (often self-limited) and keratitis have been important manifestations in patients with intact immune systems (2).

Microsporidian infection in many mammals leads to a chronic infection with persistently high antibody titers and ongoing inflammation that can reactivate, leading to acute infection when immune suppression is administered (27, 28). Studies using *Encephalitozoon* species in mice have helped define the protective mammalian immune response to microsporidiosis (29, 30). Immunity to infection is mediated by T cells and is dependent on interferon-gamma and interleukin-12 (31–34). Evidence for this is provided by the protection against lethal microsporidiosis (*Encephalitozoon cuniculi* infection) afforded by adoptive transfer of sensitized syngenic T-enriched spleen cells into athymic or SCID mice (35, 36). In intraperitoneal *E. cuniculi* infection models, mice deficient in CD8+ cells or perforin-deficient mice have a lethal infection, but there is no lethality in CD4+-deficient mice (32, 37). Oral *E. cuniculi* infection murine models have shown that CD4+ cells are also involved in the protective immune response for this natural route of infection (33, 38). The most important subset for protective immunity in oral infection models was CD8+αβ T cells, and dendritic cell interferon-gamma protection was critical for priming the gut intraepithelial lymphocyte response (39). The ability to trigger a robust T cell response against microsporidian infection is age-dependent (40). Adoptive transfer of immune B lymphocytes into athymic BALB/c (nu/nu) or SCID mice or passive transfer of hyperimmune serum is not sufficient to protect athymic mice from lethal microsporidiosis (41, 42). Antibodies do, however, provide some protection as evidenced by observations that maternal antibodies protect newborn rabbits from infection with *E. cuniculi* during the first 2 weeks of life and that antibodies to the polar tube can prevent infection of host cells (43).

There are approximately 1,400 described species of microsporidia, which are distributed into about 200 genera (2), of which the following have been demonstrated to cause infections in humans (Table 1): *Nosema* (*Nosema corneum*, renamed *Vittaforma corneae* [44]; *Nosema algerae*, reclassified initially as *Brachiola algerae* [45] and now as *Anncaliia algerae* [46]), *Pleistophora* (47), *Encephalitozoon* (48, 49), *Enterocytozoon* (24), *Septata* (50) (reclassified as *Encephalitozoon* [51]), *Trachipleistophora* (52–54), *Brachiola* (45), *Anncaliia* (46), *Tubulonosema* (55, 56), *Endoreticulatus* (57), and *Microsporidium* (48).

Costa et al. (58) have reviewed drugs that are used for the treatment of microsporidiosis in various hosts. In humans with AIDS, there is reasonable evidence from case reports that treatment with antiretroviral therapy allows the patient's immune system to control and eradicate microsporidiosis (59, 60). Albendazole, a benzimidazole that binds to β-tubulin, has activity against many species of microsporidia and is used for therapy of microsporidiosis; however, albendazole is not effective for infections due to *E. bieneusi* or *V. corneae*. The tubulin genes of both *E. bieneusi* and *V. corneae* have amino acid residues that are known to be associated with albendazole resistance (61, 62). Studies suggest that *E. bieneusi* and many other microsporidian infections are responsive to fumagillin, a water-insoluble antibiotic made by *Aspergillus fumigatus* that inhibits methionine aminiopeptidase type 2, or to synthetic analogs of fumagillin, such as TNP-470 (63, 64). Ocular infections caused by microsporidia have been treated with fumagillin bicylohexylammonium in saline. Itraconazole combined with albendazole has been used as a therapy in cases of disseminated infections caused by *Trachipleistophora* or *Anncaliia*. Nitazoxinide therapy has been effective in stopping diarrhea caused by *E. bieneusi* in patients with organ transplantation (unpublished data); however, this effect may be dependent on the immune status of the patient, because this drug had minimal efficacy in patients with AIDS with low CD4 counts (65).

MORPHOLOGY AND LIFE CYCLES

The microsporidian life cycle consists of a proliferative phase (merogony), the spore production phase (sporogony), and the mature spore or infective phase (2, 66, 67) (Fig. 1). The general features of the life cycle are as follows: (i) spores are ingested or inhaled and then germinate, resulting in extension of the polar tube and injection of the sporoplasm into the host cell cytoplasm; (ii) merogony ensues as injected sporoplasm develops into meronts (the proliferative stage), which multiply, depending on the species, by binary fission or multiple fission, forming multinucleate plasmodial forms; (iii) sporogony follows merogony, and the meront cell

Table 1 Species of microsporidia infecting humans

Species	Synonym(s)	Common sites of infection in humans	Other mammalian hosts	Reported nonmammalian hosts	Disease manifestations reported
Anncaliia algerae	*Brachiola algerae* *Nosema algerae*	Eye Muscle		Mosquitoes	Myositis, keratoconjunctivitis, cellulitis
Anncaliia connori	*Brachiola connori* *Nosema connori*	Systemic			Disseminated disease
Anncaliia vesicularum	*Brachiola vesicularum*	Muscle			Myositis
Encephalitozoon cuniculi	*Nosema cuniculi*	Systemic	Wide host range	Birds	Hepatitis, encephalitis, peritonitis, urethritis, prostatitis, nephritis, sinusitis, keratoconjunctivitis, cystitis, diarrhea, disseminated infection
Encephalitozoon hellem		Eyes	Bats	Birds	Superficial keratoconjunctivitis, sinusitis, pneumonitis, nephritis, prostatitis, urethritis, cystitis, diarrhea, disseminated infection
Encephalitozoon intestinalis	*Septata intestinalis*	Small intestine	Wide host range	Geese	Diarrhea, intestinal perforation, cholangitis, nephritis, superficial keratoconjunctivitis, disseminated infection
Enterocytozoon bieneusi		Small intestine Biliary tract	Wide host range	Birds	Diarrhea, malabsorption with wasting syndrome, cholangitis, rhinitis, bronchitis
Microsporidium africanum		Eyes			Stromal keratitis
Microsporidium ceylonensis		Eyes			Stromal keratitis
Microsporium CU (Endoreticulatus-like)		Muscle			Myositis
Nosema ocularum		Eyes			Stromal keratitis
Pleistophora ronneafiei		Muscle		Fish[a]	Myositis
Trachipleistophora anthropopthera		Eyes Systemic		Insects[a]	Encephalitis, keratitis, disseminated infection
Trachipleistophora hominis		Eyes Muscle		Mosquitoes[a]	Myositis, keratoconjunctivitis, sinusitis, encephalitis
Tubulinosema acridophagus		Muscle Systemic		Fruit fly[a]	Myositis, disseminated infection
Vittaforma corneae	*Nosema corneum*	Eyes Urinary tract			Keratoconjunctivitis, urinary tract infection

[a]Putative host(s) based on phylogeny or host relationships of other species within the genus.

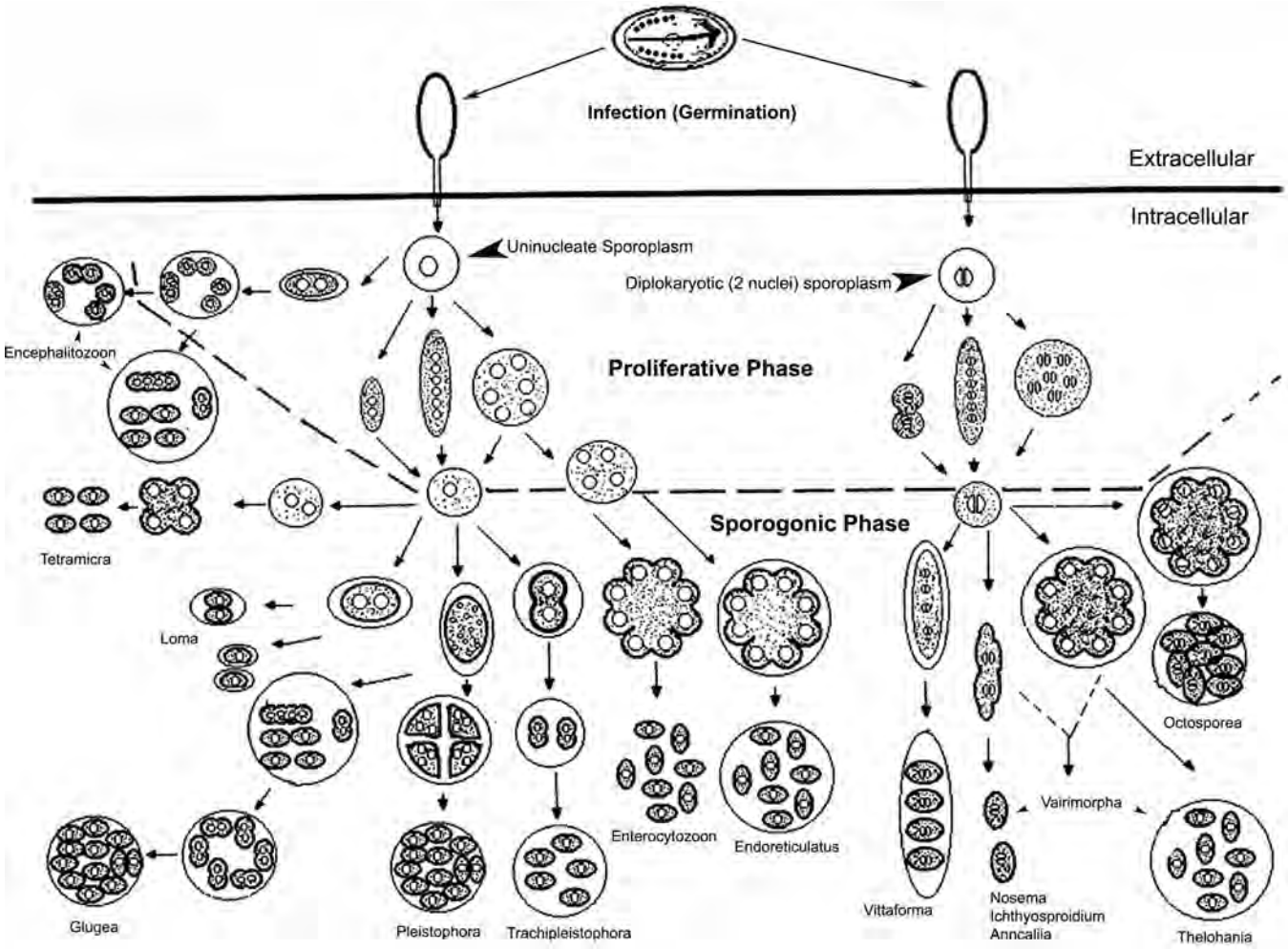


Figure 1 Microsporidian life cycles. The initial phase of infection involves spores being exposed to the proper environmental conditions that cause germination of the spores and polar tube extrusion. The polar tube pierces the plasma membrane (solid black line) of the host cell, and the sporoplasm travels through the polar tube into the host cell. The sporoplasm then divides during the proliferative phase, and the morphology of this division is used for determination of microsporidian genera. The sporoplasm on the left is uninucleate, and the cells that are produced from it represent the developmental patterns of several microsporidia with isolated nuclei. The sporoplasm on the right is diplokaryotic, and it similarly produces the various diplokaryotic developmental patterns. Cells containing either type of nucleation will produce one of three basic developmental forms. Some cycles have cells that divide immediately after karyokinesis by binary fission (e.g., *Anncaliia*). A second type forms elongated moniliform multinucleate cells that divide by multiple fission (e.g., some *Nosema* species). The third type forms rounded plasmodial multinucleate cells that divide by plasmotomy (e.g., *Endoreticulatus* species). Cells may repeat their division cycles one to several times in the proliferative phase. The intracellular stages in this phase are usually in direct contact with the host cell cytoplasm or closely abutted to the host endoplasmic reticulum; however, the proliferative cells of *Encephalitozoon* (and probably *Tetramicra*) are surrounded by a host-formed parasitophorous vacuole throughout their development, and the proliferative plasmodium of the genus *Pleistophora* is surrounded by a thick layer of parasite secretions that becomes the sporophorous vesicle in the sporogonic phase. The sporogonic phase is illustrated below the dashed line. Some of the microsporidian genera maintain direct contact with the host cell cytoplasm during sporogony, i.e., *Nosema*, *Ichthyosporidium*, *Anncaliia*, *Enterocyotozoon*, and probably *Tetramicra*. The remaining genera form a sporophorous vesicle as illustrated by the circles around developing sporogonial stages. It should be noted that in the *Thelohania* cycle and the *Thelohania*-like part of the *Vairimorpha* cycle, the diplokarya separate and continue their development as cells with isolated nuclei. Adapted with permission from reference 70.

membranes thicken, forming sporonts; (iv) after dividing, sporonts give rise to sporoblasts that develop into mature spores without additional multiplication; and (v) the host cell becomes distended with mature spores, ruptures, and releases spores into the environment. The combination of multiplication during both merogony and sporogony results in a very large number of spores being produced from a single infection and is the basis of the enormous reproductive potential of these pathogens.

Microsporidia form characteristic unicellular spores (Fig. 2) that are environmentally resistant, enabling their transmission in food and water (68). Spore structure is characteristic of the phylum, and spores can vary in size and shape depending on the species (9, 69). The spore coat consists of an electron-dense, proteinaceous exospore, an electron-lucent endospore, and an inner membrane or plasmalemma. A defining characteristic of these pathogenic protists is the presence of an invasion organelle that consists of the polar tube that is attached to the inside of the anterior end of the spore by an anchoring disk, and a series of associated membranes. The polar tube coils around the nucleus and there can be up to 30 coils depending on the species of microsporidium (2, 9, 70). During germination, the polar tube rapidly everts, forming a hollow tube that brings the sporoplasm into intimate contact with the host cell and acting as a conduit to deliver the sporoplasm to the host cell, essentially functioning like a hypodermic needle (71–73) (Fig. 3). When a spore is phagocytosed by a host cell, germination can occur, allowing the polar tube to pierce the phagocytic vacuole and permitting the sporoplasm to escape the phagosome and to be delivered into the host cell cytoplasm (74).

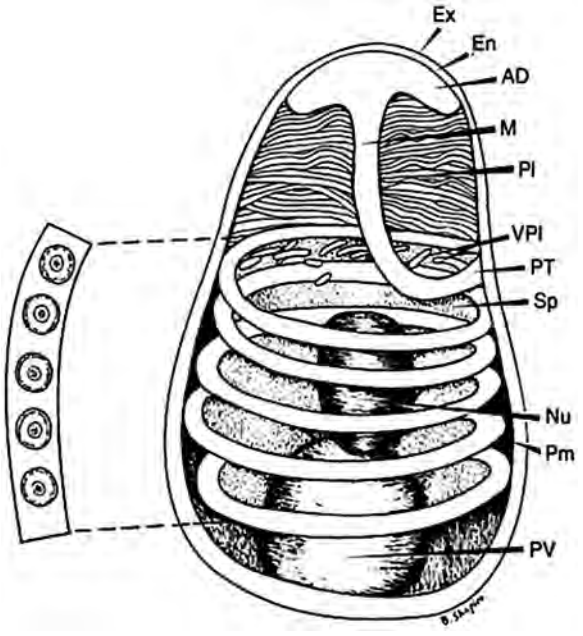


Figure 2 Diagram of a microsporidian spore. Spores range in size from 1 to 10 μm. The spore coat consists of an electron-dense exospore (Ex), an electron-lucent endospore (En), and a plasma membrane (Pm). It is thinner at the anterior end of the spore. The sporoplasm (Sp) contains a single nucleus (Nu), the posterior vacuole (PV), and ribosomes. The polar filament is attached to the anterior end of the spore by an anchoring disc (AD) and is divided into two regions: the manubroid, or straight portion (M), and the posterior region forming five coils (PT) around the sporoplasm. The manubroid polar filament is surrounded by the lamellar polaroplast (Pl) and vesicular polaroplast (VPl). The insert depicts a cross section of the polar tube coils (five coils in this spore), demonstrating the various concentric layers of different electron density and electron-dense core present in such cross sections. Reprinted with permission from reference 70.

Polar tube discharge from the anterior pole of the spore is a rapid event, and the conditions that promote germination vary widely among different microsporidia and probably reflect the adaptation of a particular microsporidium to a specific host and external environment during transmission of these pathogens (reviewed by Keohane and Weiss [75]). Conditions that promote spore discharge include pH shifts, dehydration followed by rehydration, various cations and anions, mucin or polyanions, hydrogen peroxide, ultraviolet irradiation, and calcium flux (75). In response to their particular activation signal, microsporidia have an increase in intrasporal osmotic pressure, resulting in an influx of water into the spore, which is accompanied by swelling of the polaroplasts and posterior vacuole prior to spore discharge (76, 77). In *A.* (*N.*) *algerae*, it has been suggested that this is mediated by the breakdown of trehalose by trehalase (76, 78).

PHYLOGENETIC ANALYSIS OF THE MICROSPORIDIA

Microsporidia have several apomorphic characteristics including the polar tube, the posterior vacuole, the polaroplast, and the diplokaryon (in some species) that distinguish them as a taxon. These ultrastructural features and phenotypic, developmental, and ecological characteristics have been the basis of the classical classification of these organisms. Tuzet et al. (79), Sprague (9), Larsson (80), Issi (81), Weiser (82), Sprague et al. (83), and Weiss and Becnel (2) have all provided useful overviews of the history, ultrastructural and structural characteristics, and life cycle differences of the various microsporidian taxa. Microsporidia can be divided into three main groups: (i) the "primitive" (Metchniko-



Figure 3 Scanning electron micrograph of microsporidia infection of a host cell shows the extruded polar tube of a spore of *Encephalitozoon intestinalis* piercing and infecting Vero E6 green monkey kidney cells in tissue culture. Reprinted with permission from reference 70 and with the kind permission of N. P. Kock, C. Schmetz, and J. Schottelius, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; published in Kock NP. 1998. Diagnosis of human pathogen microsporidia (dissertation).

vellidae) hyperparasites of gregarines in annelids that have a rudimentary polar filament and a spore without a polaroplast; (ii) the Chytridiopsidae, Hesseidsae, and Burkeidae, which have a short polar filament and minimal development of the polaroplast and endospore; and (c) the "higher" Microsporidia that have a well-developed polar filament, polaroplast, and posterior vacuole. The various modern classification systems for the microsporidia focus on the characteristics used to divide the third group into subgroups. These systems, however, have problems, because many of these characteristics may be convergent. For example, the characteristics of being diplokaryotic during the microsporidian life cycle and the presence of a pansporoblastic membrane are not defining characteristics, because the genus *Nosema*, defined as being diplokaryotic throughout its life cycle, is actually a polyphyletic assemblage of unrelated taxa. To this end, *Nosema locustae* has been redefined as both *Paranosema* (Sokolova et al., 2003; Sokolova et al., 2005) and *Antonospora*, (Slamovits et al., 2004); *Nosema kingi* is now *Tubulinosema kingi* (Franzen et al., 2005); *Nosema algerae* has been redefined as *A. algerae* (Franzen et al., 2006); and *Nosema cristatella* (Canning et al., 1997) is now *Pseudonosema cristatella* (Canning, 2002).

Currently over 3,000 partial and complete small subunit ribosomal RNA (ssrRNA) genes of various microsporidia species and isolates are accessible on Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and MicrosporidiaDB (http://microsporidiadb.org/micro/). This ssrRNA sequence data has been useful in the development of diagnostic PCR primers as well as for investigations into phylogenetic relationships (84, 85) (reviewed by Weiss and Vossbrinck [25]). The ssrRNA sequence of microsporidia diverges greatly from that of other eukaryotes, it is significantly shorter than other eukaryotic ssRNAs, and microsporidian ssRNA lacks loops that are present in either eukaryotic and/or prokaryotic ssrRNA (11, 86–88). This divergence of ssRNA sequence structure, while once thought to be evidence of microsporidia being an early branch in evolution, instead is due to the specialization and extremely reduced genome size reduction of these obligate intracellular pathogens (11). The genome size of the microsporidia varies from 2.3 to 51.3 Mb (89, 90). The genome sizes of microsporidia in the family *Encephalitizoonidae* (all of which are human pathogens) are all under 3.0 Mb, making these genomes among the smallest eukaryotic nuclear genomes (91–96). There are almost no introns in these compact genomes, gene density is high, proteins are shorter than the corresponding genes in *Saccharomyces cerevisiae*, and there is a high degree of gene composition conservation among the various microsporidia (91–96). Chromosomal analysis of *E. cuniculi* (97, 98) and studies of the heterogeneity of gene loci in both *E. cuniculi* (99) and *Nematocida parisii* (100) indicate that microsporidia are probably diploid. Data on *N. ceranae* have further suggested that this microsporidium is tetraploid (101). Several microsporidian genomes have been sequenced and annotated, and these data are available on MicrosporidiaDB (http://microsporidiadb.org/micro/) (102).

The development of molecular data, e.g., ssrRNA and now genomic sequencing, has resulted in the reassignment of many genera as well as the erection of new genera. It is clear that descriptions of new microsporidian species should now include detailed microscopic and ultrastructural images along with supporting molecular data, e.g., ssrRNA sequence (2, 89, 103). Studies using ssRNA have demonstrated that some microsporidia thought to be separate species are synonymous, e.g., *N. bombycis* and *Nosema tichoplusiae* (104), and confirmed that other microsporidia, e.g.,

Encephalitozoon hellem and *Encephalitozoon intestinalis*, are indeed separate species (105). A reasonable approach for integration of molecular data and other characteristics used for microsporidian classification is to create molecular phylogenies of the microsporidia and then to place the nonmolecular data on the tree (84). This type of analysis should facilitate studies that seek to understand the evolution of these organisms, including features such as the loss and gain of alternative hosts, host switching, loss and gain of sexual recombination, use of the pansporoblastic membrane, changes in the numbers of coils in the polar filament versus tissues infected, strategies of generalist versus specialist parasites, and changes in genome size and composition (103). Studies using this technique have demonstrated a significant degree of homoplasy (convergence, parallelism, and reversal) among the microsporidia, which probably reflects an effect of the reduced genome size seen in these pathogenic protists. Host-parasite cospeciation may have occurred with microsporidia and has been examined in *Nosema* species from bees (106, 107) and in the *Amblyosporidae* from mosquitoes (108, 109). Several other studies have examined the phylogeny of microsporidia isolated from various host types including fish (110, 111), insects (2, 112–114), and crustaceans (115, 116). It should be noted that discrepancies exist between phylogenies based on traditional characteristics and those based on molecular analysis and that these types of discrepancies have not been fully resolved (103).

Molecular phylogenetic data provide several lines of strong evidence that microsporidia are related to the Fungi as either a basal branch of the Fungi or a sister group (12, 13, 117). Analysis of α-tubulin, β-tubulin, glutamyl-tRNA synthetase, seryl-tRNA synthetase, vacuolar ATPase, TATA box binding protein, transcription initiation factor IIB, subunit A of vacuolar ATPase, guanosine triphosphate-binding protein, heat shock protein gene (*hsp70*), the largest subunit of RNA polymerase II (*RPB1*), and transcription factor IIB sequences demonstrates that microsporidia are not primitive eukaryotes but instead are related to fungi (117–127). Microsporidian ssrRNA genes lack a paromomycin binding site, similar to fungi (128). Microsporidian genomes display two syntenic ribosomal genes (RPL21 and RPS9) that are also syntenic throughout the fungi but are linked in other eukaryotic lineages (129, 130). The microsporidian EF-1α gene has an insertion that is found only in fungi and animals—not in protozoa (12, 128, 131, 132). Analysis of the *E. cuniculi* genome and other microsporidian genomes demonstrates that most microsporidian proteins are most similar to fungal homologs (13, 14, 91, 95, 96, 133). Biological evidence also supports the relationship of microsporidia to fungi. Microsporidia have chitin in their spore wall and store trehalose, as do fungi (69, 78). Microsporidia display similarities to the fungi during mitosis (e.g., closed mitosis and spindle pole bodies [134]) and meiosis (135). Data suggest that trehalose is probably the major sugar reserve in microsporidia, as is seen in many fungi (2). *O*-mannosylation, as seen in fungi, occurs in microsporidia as demonstrated by studies of major polar tube protein PTP1 (136).

Keeling (137), in an analysis of β-tubulin data that included microsporidia and representatives of many fungal phyla, found evidence that microsporidia were a sister group to the Zygomycota. Comparative analysis utilizing the complete genomes of several microsporidia also supports a similar relationship of microsporidia with the Fungi (13, 92, 130) Analysis of a six-gene dataset (16) that included a large diversity of Fungi suggested that microsporidia branched deeply, forming a sister clade to the Fungi with *Rozella*, a hyperparasitic chytrid that infects other chytrids. Analyses of individual gene trees, supertrees, and trees based on concatenation of hundreds of genes also supported a deep-branching position as a sister group to the Fungi (13, 138). However, these data, while suggestive, do not definitively determine if the microsporidia (and *Rozella*) are within the Fungi or sisters to the Fungi. Environmental microbiome studies have defined a previously unrecognized group of organisms termed the Cryptomycota (139–143), which are a large and diverse lineage, including aphelids and *Rozella*, of Fungi or fungal relatives that are poorly described at the biological level but that are closely related to the microsporidia. The most recent genome-scale phylogenies place microsporidia together with Cryptomycota as the basal branch of the fungal kingdom (or alternatively as a sister phylum). These, and other, recently described microsporidia-like organisms, e.g., *Mitosporidium daphniae* (144), *Nucleophaga terricolae*, and *Paramicrosporidium* (15, 145, 146), illustrate that more environmental sampling and genome sequencing will be needed to fully resolve the relationship of microsporidia and the Fungi, as well as to provide a better understanding of the Cryptomycota and reveal what this group has to teach us about the origin and diversification of the microsporidia.

STRUCTURE AND COMPOSITON OF THE MICROSPORIDIAN SPORE

The spore is the infectious stage of the microsporidian life cycle and the only stage that can survive outside the

host cell. The shape of the microsporidian spore is usually pyriform or oval, and the spores vary in size from approximately 1 to 12 μm, although some needle-like spores can be as long as 20 μm (9). The spore has three general features: the spore coat, the sporoplasm, and the invasion apparatus (147). The spore coat contains three layers: an electron-dense outer exospore layer, an electron-lucent inner endospore layer, and the plasma membrane (147). The endospore layer is composed predominantly of chitin and glycoproteins, and it is thinnest in the region of the anterior anchoring disk complex where rupture occurs during extrusion of the polar tube. Chitin, a major component of the endospore, forms bridges across the endospore and is part of the fibrillar system of the exospore (69). The presence of chitin in the endospore enables microsporidian spores to be stained by fluorescent brighteners such as calcofluor white or Uvitex 2B (148). Both chitin deactylase, in several microsporidia (149), and a *N. bombycis* GH19 chitinase were found to localize to the spore wall (150).

The spore wall protects the organism from harsh environmental conditions, permitting transmission of the organism via water or food. It is probable that proteins in the spore wall are involved in processes such as spore adherence, signaling, or other interactions with host cells (151). Increased hydrostatic pressure in the spore has been demonstrated to cause discharge of the polar tube (152). Several spore wall proteins have been identified using proteomic techniques, and the localization of these proteins has been validated by immunofluorescence (indirect fluorescent-antibody assay) and ultrastructural (immunoEM) studies. The first spore wall protein (SWP) to be identified was *E. cuniculi* spore wall protein 1 (EcSWP1) (153). Ultrastructural analysis has demonstrated that this protein is found in the exospore. During spore development EcSWP1 was absent in meronts and was first seen in early sporonts, and the amount of this protein increased in the exospore during spore maturation (153). Homologs of SWP1 have been identified in *E. intestinalis* and *E. hellem* (154, 155). In *E. hellem* two SWP1 proteins (EhSWP1a, EhSWP1b) were identified. Using monoclonal antibodies to various SWP1 proteins, EiSWP1, EhSWP1a, and EhSWP1b were all found to localize to the exospore in mature spores (154, 155). A second SWP protein (SWP2) was identified in *E. intestinalis* along with EiSWP1. This 150-kDa protein shared a high similarity with the N-terminal region of EiSWP1; immuno-EM demonstrated that EiSWP2 localized to the spore surface and that EiSWP2 became much more abundant as spores continued development into sporoblasts and mature spores. Homologs of EiSWP2 have not been found in *E. hellem* or *E. cuniculi*, suggesting that this protein may have a unique role in *E. intestinalis*. A third spore wall protein (SWP3/EnP2) was identified from *E. cuniculi* using a proteomic strategy (156, 157). EcSWP3, also called endospore protein 2 (EnP2), has been demonstrated to localize to the endospore adjacent to the plasma membrane (156). Another endospore protein (EnP1) was identified at the same time as SWP3/EnP2 in *E. cuniculi* (156). EnP1, which is also called SWP4, is a cysteine-rich protein and contains disulfide linkages that may facilitate its binding in the endospore.

It has been found that host cell surface sulfated glycosaminoglycans play an important role in the adherence of *E. intestinalis* and *E. cuniculi* to host cell surfaces (158). EnP1 has been found to be involved in host cell adherence by its interacting with host cell membrane glycosaminoglycans through the heparin binding motifs found in EnP1 (151). Homologs of these proteins have been found in other microsporidia. These SWP adhesion domains may facilitate the binding of microsporidian spores to either the cell surface or mucus of the gastrointestinal tract prior to germination. Many of the SWPs that have been identified in various microsporidia have posttranslational glycosylation, often involving mannosylation, which is likely important for spore wall adherence to host cells during invasion. Using various proteomic techniques, additional SWPs such as SWP5 (159), SWP6, and SWP7 (L. M. Weiss, unpublished data) have also been identified in the *Encephalitozoonidae*, and other putative SWPs have been identified in *N. bombycis* (160–164). Some *N. bombycis* SWPs have been suggested to function as scaffolding proteins that support other proteins to form the integrated spore wall of microsporidia (162). Many of these newly identified *N. bombycis* SWPs have not been found in the *Encephalitozoonidae*, suggesting (not surprisingly) that some SWPs may be species (or genus) specific and related to the environment and the specific interactions that are needed for infection between different hosts (e.g., insects versus mammalian hosts). SWPs appear to be specific to microsporidia, because homologs of these various SWPs have not been found in other organisms.

STRUCTURE AND COMPOSITION OF THE POLAR TUBE

It has been almost 125 years since the initial description of polar tubes and their discharge from spores (165, 166). The polar tube is an invasion organelle that is unique to the microsporidia. While the polar

tube superficially resembles the structures seen in Myxosporea (which are related to cnidarians), the microsporidian polar tube has a distinct function, acting as a tube rather than as a nematocyst. During infection this highly specialized structure is discharged from the spore, forming a hollow tube that delivers the infectious sporoplasm into its host cell's cytoplasm. The polar tube in different species of microsporidia is variable in diameter, length, and arrangement in the spore. The number of coils in a spore can range from 3 to 50 depending on the species of microsporidia (167). Polar tubes can be from 50 to 500 μm in length, and the length can be two to three times longer after the polar tube has completely discharged from the spore (73). The diameter of the tube is 0.1 to 0.2 μm, and during sporoplasm passage the diameter can increase significantly, illustrating the flexibility of this structure (73, 168, 169).

The mechanism of spore germination still remains to be definitively determined. It is generally believed that spore germination occurs in several phases that may overlap: (i) activation, (ii) an increase in intrasporal osmotic pressure, (iii) eversion of the polar tube, and (iv) passage of the sporoplasm through the everted polar tube (2). Before germination, the polar tube is coiled inside the mature spores and filled with material when visualized by transmission electron microscopy. After activation, the conditions of which are often specific to a particular species of microsporidia, the polar tube bursts through the anterior pole of spore at the location of the anchoring disk (73, 170) and forms a hollow tube (170, 171). This discharge process occurs rapidly, with full eversion occurring within 2 s (71). Different species of microsporidia require different conditions for germination, and this probably depends on the host species target as well as environmental conditions related to transmission of a particular organism. While a universal stimulant for spore activation is not known, many species can be induced to germinate in distilled water containing 1 to 5% hydrogen peroxide (75). Various reports using *A.* (*N.*) *algerae* have demonstrated that successful germination is associated with an increase in intrasporal osmotic pressure and that this osmotic pressure is the force that drives polar tube extrusion, rather than this requiring ATP or another energy source (152). These studies of *A. algerae* found that the increase in osmotic pressure was related to a decrease in trehalose levels in germinating spores. This suggests that upon activation of spores, the breakdown in compartmentalization brings trehalose in contact with the enzyme trehalase, resulting in its digestion into simple sugars increasing spore osmotic pressure (172).

Proteomic studies of the polar tube have begun to define the full complement of proteins that make up this structure, which should lead to a better understanding of how the polar tube is formed and how it functions during invasion (136, 173–188). Polar tube proteins (PTPs) are highly immunogenic in both experimental and natural infections. In a large serosurvey, antibodies reacting to *E. cuniculi* were present in 5% of pregnant French women and 8% of Dutch blood donors (189). Polar tubes have been found to be insoluble in 1% SDS and 9 M urea and soluble in 2% dithiothreitol (DTT), allowing the purification of potential PTPs. Weidner used these solubility properties to isolate potential PTPs from *Ameson michaelis* and demonstrated that this protein mixture could self-polymerize (169). Using a similar extraction procedure and reverse-phase high-performance liquid chromatography, a 43-kDa PTP was identified and designated PTP1, based on its being the major protein by mass in the *Glugea americanus* DTT solubilized material (174). Subsequently, additional PTP1s were purified from several microsporidia of the genus *Encephalitozoonidae* using the same purification protocol (175, 177, 190).

PTP1 is a proline-rich protein that contains many cysteine residues in its N-terminal and C-terminal domains. Based on the solubilization of the polar tube by DTT, disulfide bridges probably play an essential role in maintaining this structure. The abundance of proline in PTP1 is consistent with properties seen in the intact polar tube because this amino acid has been reported to increase the tensile strength and elasticity of proteins. Analysis of the glycosylation of PTP1 demonstrated that there are a significant number of O-linked mannosylation sites in this protein (136, 183, 186). Mannose pretreatment of host cells was found to decrease *E. hellem* infection, which was consistent with an interaction between mannosylated PTP1 and some unknown host cell mannose-binding receptor protein (136, 184, 185, 191, 192). Examination of published microsporidian genomes (www.MicrosporidiaDB.org) has demonstrated that PTP1 homologs are not easily identified in all genomes, which may reflect the high evolution rates in microsporidia that often make the identification of orthologous proteins difficult. For example, the putative PTP1 of *Antonospora locustae*, *Paranosema grylli*, and *N. ceranae* were identified based on a combination of features including the presence of a predicted signal peptide, their similar length, conserved acidic pI, and predicted amino acid compositions along with their close physical juxtaposition to another PTP, named PTP2 (180); but it would not have been identified by a simple BLAST search of the genomes (2, 193–195).

PTP2 was also identified using proteomic and antibody-based approaches (180, 184, 196). Interestingly, PTP2 is found at the same genomic locus as PTP1. Preservation of gene order may be due to constraints of evolution on these small eukaryotic genomes (197). PTP2 is more conserved in size than PTP1, and the identified PTP2s from various microsporidia share common characteristics, despite a high degree of sequence divergence; e.g., they have a basic pI, high lysine content, and a conservation of cysteine residues that are most likely involved in intra- and/or inter-protein disulfide bridges (180, 184, 196). PTP2 has been identified in the genome of many microsporidia including *A.* (*Paranosema*) *locustae*, *Trachipleistophora hominis*, *E. cuniculi*, *E. hellem*, *E. intestinalis*, *P. grylli*, *N. cerana*, *N. bombycis*, *E. bieneusi*, *A. algerae*, *Tubulinosema ratisbonensis*, *N. parisii*, *V. cornea*, and *Edhazardia aedis* (2, 184, 194, 196).

Immunoscreening of a cDNA library of *E. cuniculi* led to the identification of a third polar tube protein, PTP3 (182). Unlike PTP1 and PTP2, it was found that PTP3 is solubilized in the presence of SDS alone without the need for a reducing agent such as DTT (180), which explains why it was not seen in standard polar tube preparations. PTP3 along with PTP1 and PTP2 was, however, found in studies examining cross-linked polar tube complexes, and these three PTPs were also demonstrated to interact in yeast two-hybrid assays (182, 185). It has been suggested that PTP3 may act as a scaffolding protein for the assembly of other PTPs in the formation of the polar tube during development. Genome analysis has confirmed the presence of PTP3 orthologs in the genomes of *E. intestinalis*, *E. hellem*, *Encephalitozoon romalae*, *N. ceranae*, *N. bombycis, A. locustae, P. grylli, T. hominis*, *E. bieneusi*, *A. algerae*, *N. parisii*, *Octosporea bayeri*, *T. ratisbonensis*, and *Vavria culicis floridensis* (2). The sequence of these PTP3 orthologs is more conserved than the sequences of the PTP1 and PTP2 orthologs.

Two new PTPs (PTP4 and PTP5) have been identified in the polar tube using proteomic and antibody-based approaches (198). Orthologous PTP4 and PTP5 are present in many microsporidian genomes including *E. cuniculi*, *E. hellem*, *E. intestinalis*, *P. grylli*, *N. ceranae*, *N. bombycis*, *A. algerae*, *O. bayeri*, *E. aedis*, *V. cornea*, and *T. ratisbonensis* (2). Similar to PTP1 and PTP2, the genes for PTP4 and PTP5 usually are in a cluster in the genome (2). Monoclonal antibody to PTP4 stains the extruded tip of the polar tube, whereas the other PTPs (i.e., PTP1, PTP2, PTP3, PTP5) are located uniformly along the entire polar tube (B. Han and L. M. Weiss, submitted) (2, 196). This specialized location of PTP4 suggests that this protein is probably involved in the interaction of the polar tube and host cell at the invasion synapse. Recently, by employing PTP4 for an immunoprecipitation assay followed by proteomic analysis, we were able to identify a potential host cell receptor for PTP4, and mutant host cells without this receptor have a markedly reduced infection rate (Han and Weiss, submitted). The mechanism by which the polar tube interacts with the host cell membrane resulting in penetration is currently unknown; however, there is some evidence that host cell actin may be involved in microsporidian penetration of the host cell within the invasion synapse (199). Furthermore, ongoing proteomic studies of the polar tube suggest that additional PTPs remain to be characterized in this structure (L. M. Weiss, unpublished data).

SUMMARY

Microsporidia are unicellular eukaryotes that develop as obligate intracellular parasites. They have a unique invasion organelle, the polar tube, which upon appropriate environmental stimulation rapidly discharges out of the spore, pierces a cell membrane, and serves as a conduit for sporoplasm passage into a host cell. Phylogenetic analysis suggests that microsporidia are related to the Fungi, being either a basal branch or sister group. Despite the description of microsporidia over 150 years ago, we still lack an understanding of the mechanism of invasion, including the role of various PTPs and SWPs as well as host cell proteins in the formation and function of the invasion synapse. Over the past 2 decades, proteomic approaches have helped define PTPs and SWPs as well as the importance of post-translational modifications such as glycosylation in the functioning of these proteins, but the absence of genetic techniques for the manipulation of microsporidia has hampered research on the function of these various proteins. Recent advances in ultrastructural techniques are, however, helping to better define the role of various PTPs and SWPs in the formation and functioning of the invasion synapse. The study of the mechanism of invasion should provide fundamental insights into the biology of these ubiquitous intracellular pathogens that can be integrated into studies aimed at treating or controlling microsporidiosis.

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Life of Fungi

II

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Fungal Sex: The *Ascomycota*

6

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PHYLOGENY OF THE ASCOMYCETES

There are ~64,000 known species within the *Ascomycota*, making it the largest phylum of Fungi. Major subphyla include the *Taphrinomycotina* (e.g., *Schizosaccharomyces pombe*), the *Saccharomycotina* (including *Candida* and *Saccharomyces* clades), and the *Pezizomycotina* (the largest subphylum, which includes the *Eurotiomycetes*, *Dothideomycetes*, *Sordariomycetes*, and *Leotiomycetes*) (see Fig. 1). Most *Saccharomycotina* grow as budding yeast or are dimorphic (can grow as yeast or filaments), whereas most *Pezizomycotina* are predominantly filamentous, although some are also dimorphic.

SEX IN THE MODEL YEASTS

Sexual reproduction is thought to have evolved early in the eukaryotic lineage and to have been retained by the majority of extant species. The unicellular yeasts *Saccharomyces cerevisiae* and *S. pombe* have been invaluable in defining fundamental aspects of sexual reproduction, as well as addressing the benefits of sex for adaptive evolution (1, 2). Despite often being grouped together, *S. cerevisiae* (a budding yeast) and *S. pombe* (a fission yeast) last shared a common ancestor more than 330 million years ago (3). The *Schizosaccharomyces* genus forms a deep branch from the base of the *Ascomycota* tree (the *Taphrinomycotina*) and is considered to be representative of "ancient" ascomycetes (4) (Fig. 1). *S. cerevisiae* has undergone faster evolution than *S. pombe* since their split from a common ancestor, so that the latter may display attributes closer to the common roots of fungi and animals (4). The two yeasts exhibit both similarities and important differences in mating (gamete fusion) and meiosis (gametogenesis). Mating in these species involves pheromone-based communication and fusion between cells of opposite mating types. In *S. cerevisiae*, signaling between **a** and α cell types leads to the formation of diploid **a**/α cells, whereas in *S. pombe*, mating of P (plus or h+) and M (minus or h–) cells generates diploid P/M (h+/h–) cells (Fig. 2).

Regulation of Cell Type Identity

Genes encoded at the mating-type (or *MAT*) locus define cell type identity. In *S. cerevisiae*, haploid *MAT***a** cells express the *MAT**a**1* gene, haploid *MAT*α cells express *MAT1* and *MAT2* genes, and diploid *MAT***a**/α cells express all three *MAT* genes (*5*, 6). Both *MAT**a**1* and *MAT2* encode homeodomain proteins (**a**1 and α2, respectively), whereas *MAT1* encodes an α-domain protein (α1). These activities cooperate to ensure that haploid **a** and α cells express sex-specific pheromones and pheromone receptors. Furthermore, they allow **a** and α cells to express "haploid-specific" genes, which include several components of the pheromone-signaling mitogen-activated protein kinase (MAPK) cascade.

In *S. cerevisiae* α cells, α-specific genes are expressed due to α1 and Mcm1 activity, and the expression of **a**-specific genes is repressed by α2/Mcm1. In **a** cells, **a**-specific genes are constitutively expressed due to the presence of Mcm1 (a constitutive MADS-box transcription factor), while α-specific genes are off due to the absence of α1. In diploid **a**/α cells, both **a**- and α-specific genes are turned off, because a complex between **a**1 and α2 represses expression of α1 (and hence α-specific genes), while **a**-specific genes are repressed by α2/Mcm1. Critically, the **a**1/α2 complex also represses "haploid-specific" genes; because these genes include inhibitors of meiosis, the requirement for the **a**1/α2 complex ensures that only diploid **a**/α cells can initiate meiosis.

In *S. pombe*, a similar mechanism regulates cell type identity, with cells expressing the *mat1-P* locus mating as P cells and *mat1-M*-expressing cells mating as M cells. The *mat1-P* locus encodes Pc and Pi proteins,

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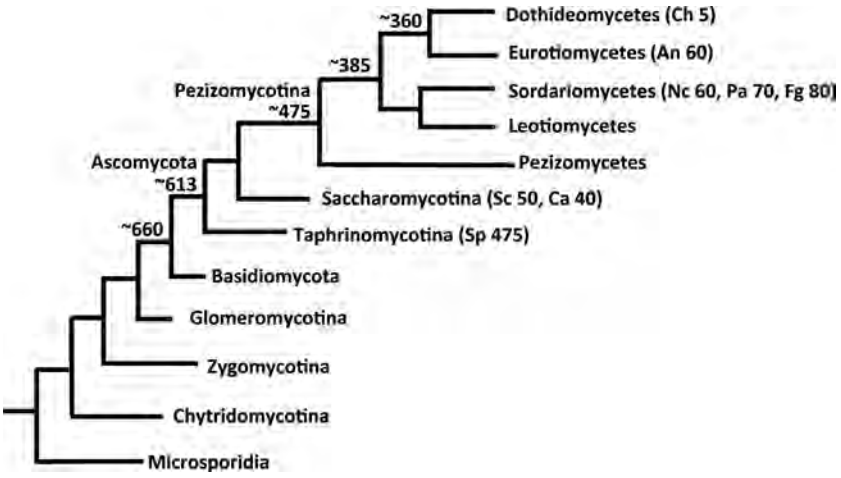


Figure 1 Phylogenetic relationships of major groups of fungi. Synthesis from references 280–282. Numbers at the nodes indicate estimated age, in millions of years, at which an ancestral group arose. Abbreviations: An, *Aspergillus nidulans*; Ca, *Candida albicans*; Ch, *Cochliobolus heterostrophus*; Fg, *Fusarium graminearum*; Pa, *Podospora anserina*; Nc, *Neurospora crassa*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*. Numbers in parentheses indicate the approximate age of that group in millions of years.

whereas the *mat1-M* locus encodes Mc and Mi proteins. For successful mating, the *mat1-Pc* gene is required for expression of P-specific genes, whereas the *mat1-Mc* gene drives expression of M-specific genes (7). As in *S. cerevisiae*, cell-type-specific expression includes that of pheromone and pheromone receptor genes (8–11). While *mat1-Pc* and *mat1-Mc* genes are sufficient for conjugation, expression of all four *mat1*-encoded proteins is necessary for *S. pombe* meiosis.

Pheromone Signaling in *S. cerevisiae*

Pheromone signaling in fungi is transduced via a conserved MAPK pathway (see Fig. 3) (for detailed reviews see references 12–15). In *S. cerevisiae*, pheromone binding to its cell surface receptor activates a heterotrimeric G protein complex, which dissociates into separate Gα (Gpa1) and Gβγ (Ste4–Ste18) components. The activated Gβγ complex directly contacts several components: (i) Ste20, a p21-activated protein kinase, (ii) Ste5,

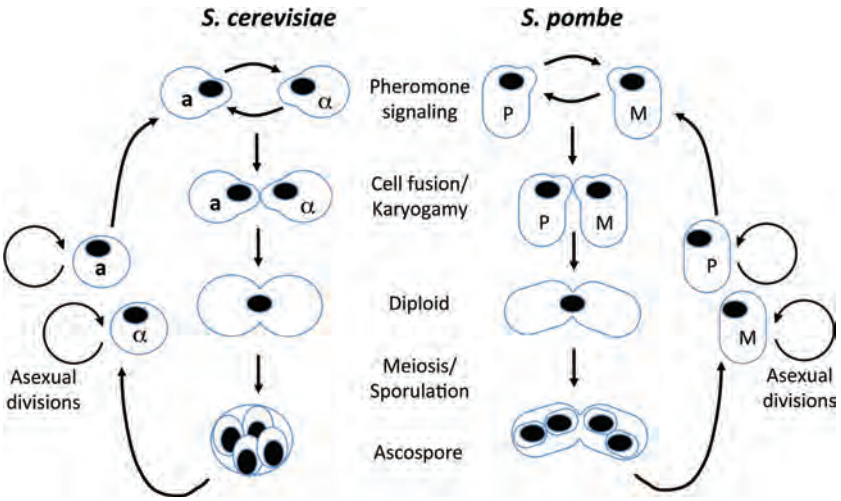


Figure 2 Life cycles of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Both species can divide asexually or can undergo opposite sex mating. Meiosis and sporulation is used to complete the life cycle and regenerate haploid forms of the species.

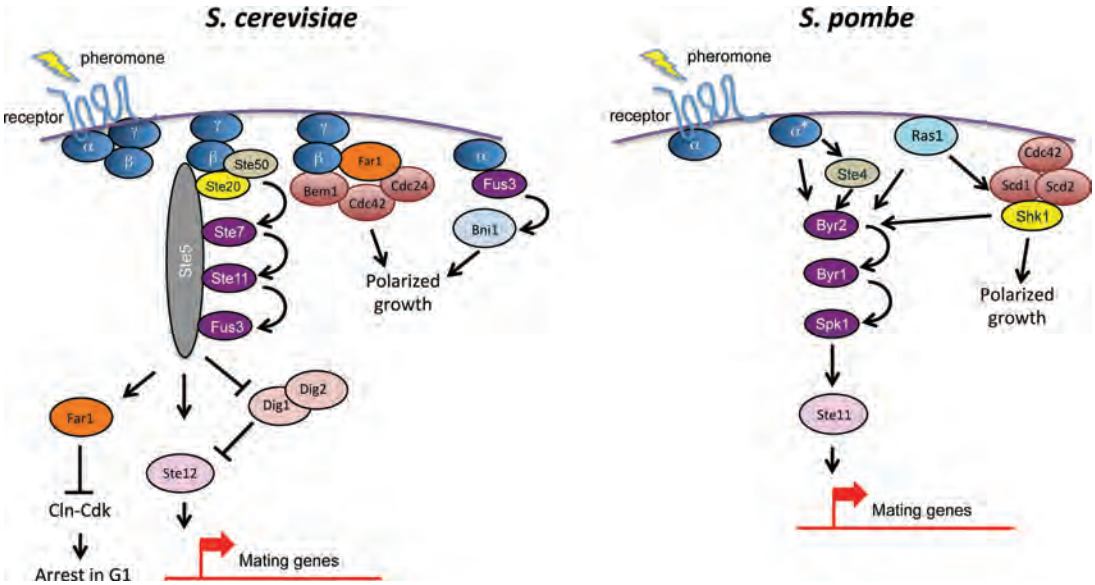


Figure 3 Pheromone signaling in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Pheromone signaling is transduced from a G-protein coupled receptor via a mitogen-activated protein kinase (MAPK) cascade into a transcriptional response in the nucleus. In *S. cerevisiae*, pheromone-receptor interactions cause dissociation of the G protein complex, and Gβγ subunits promote pheromone signaling via two scaffold proteins. The Ste5 scaffold mediates MAPK signaling and the transcriptional response to pheromone, whereas the Far1 scaffold interacts with Cdc42 to mediate shmoo formation and also leads to cell cycle arrest. In *S. pombe*, no scaffold protein has been identified for pheromone signaling. Here, the Gα subunit transduces the pheromone signal to the MAPK cascade and does so in concert with Ste4 and Ras1 activities.

a scaffold protein for MAPK proteins, (iii) Far1, a second scaffold protein involved in cell cycle arrest and cell polarization (16–19), and (iv) the Cdc42-guanine-nucleotide exchange factor, Cdc24.

Ste20 acts upstream of the MAPK cascade and is activated by the GTP-bound form of the G protein Cdc42. Because Cdc42 is membrane localized, this step recruits Ste20 to the membrane and promotes its interaction with the membrane-bound Gβγ complex (20). Cdc42 is itself activated by pheromone signaling; the Gβγ complex recruits Far1 together with Cdc24, a guanine-nucleotide exchange factor that increases the exchange of GDP for GTP on Cdc42 (16, 18). Cdc24 also functions in a Far1-independent manner by binding directly to the Gβγ complex (21). Notably, loss of Far1 affects shmoo formation and cell cycle arrest, but not the transcriptional response to pheromone (22, 23).

At the heart of the pheromone-signaling cascade is the scaffold protein Ste5, which interacts with the MAPKKK Ste11, the MAPKK Ste7, and the terminal MAPK Fus3 (24–26). Ste5 also binds the Gβ subunit, Ste4, and the guanine-nucleotide exchange factor protein, Cdc24 (19, 26). Shuttling of Ste5 between the nucleus and the cytoplasm occurs in vegetative cells, but increased export of the nuclear pool follows pheromone exposure, and Ste5 accumulates at the membrane via interactions with Gβ and Cdc24 (21, 27). The Ste5 scaffold promotes Ste11-Ste7-Fus3 interactions, and an additional contact with Gβγ brings these proteins into close proximity with the upstream kinase, Ste20. Membrane localization of Ste5 is critical for pheromone signaling; tethering of Ste5 to the membrane activates signaling even in the absence of pheromone and independently of Gβγ (28). However, signaling is still dependent on Ste20, indicating that recruitment of Ste5 to the membrane brings the Ste20 kinase into close proximity with its target, the MAPKKK Ste11 (28). Ste11 activation is also promoted by Ste50, which may promote a conformational change in Ste11 that enhances its phosphorylation by Ste20 (29). The Ste5 scaffold binds to the three kinases (Ste11, Ste7, and Fus3) via distinct interaction domains (24) and also is a direct coactivator of Fus3 phosphorylation by Ste7 (30).

Phosphorylated Fus3 protein is released from the Ste5 scaffold and enters the nucleus, where it activates the transcription factor Ste12. Fus3 directly stimulates Ste12 activity by phosphorylation and also targets the Ste12 regulators Dig1/Dig2 (31, 32). Ste12 binds DNA

as a homodimer (33, 34), as a heterodimer with Mcm1 or α1 (35–37), and as a complex with Dig1/Dig2 or Dig1/Tec1 (38). Activated Fus3 also phosphorylates and activates the Far1 scaffold protein (39). Far1 mediates cell cycle arrest by interfering with cyclin-Cdk activity, potentially by disrupting docking interactions between cyclins and their substrates (40). Far1 also acts as a scaffold for Gβγ and Cdc24, thereby establishing the site for formation of the mating projection (or shmoo) and recruiting Cdc42 and Bem1 proteins away from bud sites (23, 41). In addition to Far1, activated Fus3 also contributes to polarized growth by interacting with the free Gα subunit and promoting phosphorylation of Bni1 (42, 43). The latter results in activation of Bni1, facilitating local actin assembly and polarized growth.

Pheromone Signaling in *S. pombe*

In *S. pombe*, pheromones secreted by P and M cells bind to the cell surface receptors Mam2 and Map3, respectively. However, whereas Gβγ is responsible for MAPK signaling in *S. cerevisiae*, the Gα protein mediates MAPK signaling in *S. pombe*, and a corresponding Gβγ complex has not been identified (44). In *Kluyveromyces lactis*, the Gα subunit alone is also sufficient for signal transduction (45), whereas in *Candida albicans*, both Gα and Gβγ components make positive contributions to MAPK signaling (46).

The *S. pombe* MAPK cascade involves sequential activation of a MAPKKK (Byr2), a MAPKK (Byr1), and a MAPK (Spk1) (47, 48), and expression of these kinases can substitute for their *S. cerevisiae* counterparts and vice versa (49). MAPK signaling activates the Ste11 transcription factor, which is responsible for mating gene expression (50). Unlike *S. cerevisiae*, however, there is apparently no equivalent of the scaffold protein, Ste5, and it is not clear how the *S. pombe* Gα protein passes its signal to the MAPKKK, Byr2. One possible candidate is Ste4 (homologous to *Sc*Ste50), which interacts with both Gα and Byr2 (51, 52). The GTPase Ras1 is also necessary for *S. pombe* mating and may promote activation of the Byr2 kinase (48, 53, 54). Mating in *S. pombe* is closely integrated with nutritional cues, with nitrogen starvation leading to repression of TORC1 and cAMP pathways, and promoting basal activation of the Ste11 transcription factor (55). *S. pombe* also undergoes polarized growth in response to pheromone and, similar to *S. cerevisiae*, Cdc42 is a major regulator of cell polarity. However, it is not known how *S. pombe* Cdc42 is recruited to pheromone receptors. Ras1 could potentially link the polarization machinery to the pheromone-sensing pathway (15).

Why does *S. pombe* apparently lack the protein scaffolds central to pheromone signaling in other yeast species? One possibility is that scaffold proteins remain to be identified in *S. pombe*. Alternatively, it is possible that scaffolds evolved to limit cross talk between different signaling pathways (56–58), and this is not necessary in *S. pombe* because pheromone MAPK components are not shared with other programs. Furthermore, a major function of Far1 is to mediate cell cycle arrest in *S. cerevisiae*, whereas mating in *S. pombe* occurs in nutritionally poor conditions that may naturally arrest cells in G1. Far1 and Ste5 could therefore have become redundant in the *Schizosaccharomyces* lineage, whereas at least one of these scaffolds has been retained by other ascomycetes and basidiomycetes (15).

Meiosis in *S. cerevisiae*

Diploid **a**/α cells of *S. cerevisiae* stably propagate in the diploid state unless induced by nutrient-poor environments to enter meiosis, resulting in the formation of four haploid ascospores (see tetrads in Fig. 4). The master regulator of meiosis is the transcription factor Ime1 (inducer of meiosis 1) (59), whose expression is regulated by multiple environmental cues (60). *IME1* is also regulated by a long noncoding RNA (lncRNA), *IRT1*. This lncRNA is the result of Rme1 (repressor of Ime1) binding to the *IME1* promoter, and it induces a repressive chromatin state over the *IME1* promoter (61). In diploid cells, the **a**1/α2 complex represses *RME1* so that its gene product no longer produces the inhibitory *IRT1* and expression of *IME1* is permitted. Interestingly, a second lncRNA, *RME2*, controls expression of the *IME4* gene, which also promotes entry into meiosis (62, 63). In haploid **a** or α cells, *RME2* is an antisense transcript that blocks *IME4* expression, and inhibition is relieved in diploid cells due to **a**1/α2 blocking formation of the *RME2* transcript (64, 65). Thus, two lncRNAs, *IRT1* and *RME2*, combine to provide precise regulation over entry into meiosis (Fig. 5A).

Meiosis is accompanied by synchronous waves of gene expression involving ~1,600 genes (66–68). Ime1 may induce early meiosis genes by acting with a transcriptional cofactor, Ume6 (69–71), although a recent study suggests that Ime1 promotes Ume6 degradation to promote progression into meiosis (72). *IME2* is an early meiosis gene that encodes a serine-threonine kinase critical for downstream signaling. This kinase also targets Ime1 for destruction, thereby ensuring that Ime1 is only active in a narrow temporal window (73, 74). The Ndt80 transcription factor is the key regulator of middle meiosis genes, binding to middle sporulation

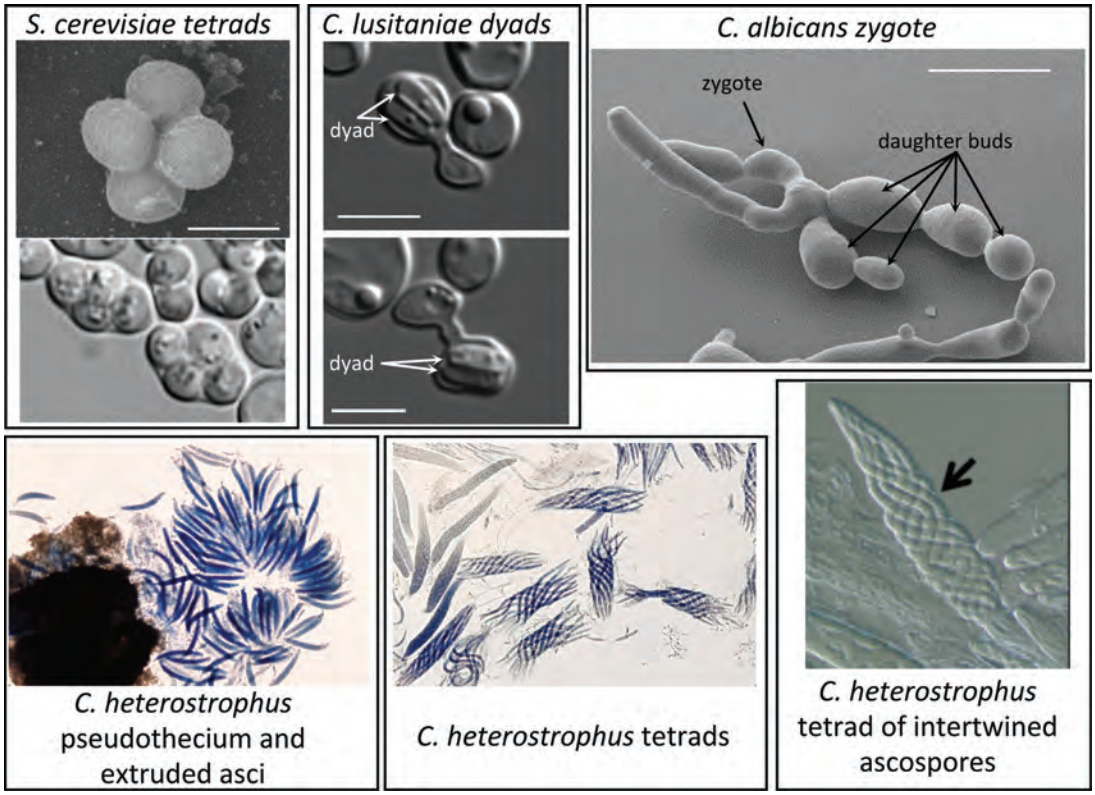


Figure 4 Images of ascomycetes undergoing sexual reproduction. The top row of images shows *Saccharomyces cerevisiae* tetrads (four ascospores) produced by meiosis, *Candida lusitaniae* dyads (two cell ascospores) produced by meiosis within the mating zygote, and a *Candida albicans* mating zygote with attached daughter cell buds. The bottom row of images shows a pseudothecium with extruded asci from the self-incompatible species *Cochliobolus heterostrophus*, *C. heterostrophus* tetrads, and *C. heterostrophus* asci containing filamentous ascospores. Scale bars in the top three panels are 3.4 m, 5 m, and 8.5 m, respectively. We acknowledge Aaron Neiman (Stony Brook University) and Matthew Hirakawa (Brown University) for the images of *S. cerevisiae* and *C. albicans* cells, respectively.

elements and removing the repressor Sum1 from middle sporulation element sequences (75). High-level expression of Ndt80 ensures commitment to the meiotic program (76).

Meiosis in *S. pombe*

S. cerevisiae cells stably propagate in the diploid state, whereas *S. pombe* cells often enter meiosis immediately after mating. Constitutive pheromone signaling is sufficient to drive *S. pombe* cells into meiosis (50). The MAPK target, Ste11, is a key transcriptional regulator of both mating and meiosis; it induces *mat1-Pc* and *mat1-Mc*, which in turn activate expression of *mat1-Pm* and *mat1-Mm* genes (77). Mat1-Pm and Mat1-Mm act synergistically to drive expression of the *mei3* gene that initiates the meiotic cascade (78, 79).

lncRNAs play a central role in regulating meiosis in *S. pombe* as in *S. cerevisiae* (Fig. 5B). Here, Mei2 recognizes an lncRNA (meiRNA) produced at the *sme2* locus and acts to sequester a key meiosis silencing factor, the RNA-binding protein Mmi1. In vegetative cells, Mmi1 silences the expression of ~20 meiosis genes by targeting their mRNAs for degradation (80–82). Sequestration of Mmi1 by Mei2 (and meiRNA) therefore allows meiotic gene expression to take place. Target genes include *mei4*, whose gene product is a transcription factor responsible for the induction of more than 500 meiosis genes (83).

Mei2 is itself regulated by the serine-threonine kinase Pat1 and the MAPK transcription factor Ste11 (Fig. 5B). Pat1 is an inhibitor of Mei2, but in diploid P/M cells the pseudo-substrate Mei3 is produced, which inhibits Pat1 kinase activity, thereby releasing Mei2 (77, 78, 84, 85). In addition, nitrogen starvation facilitates Ste11 expression, which induces the expression of Mei2 (86). As a result of these mechanisms, Mei2 is

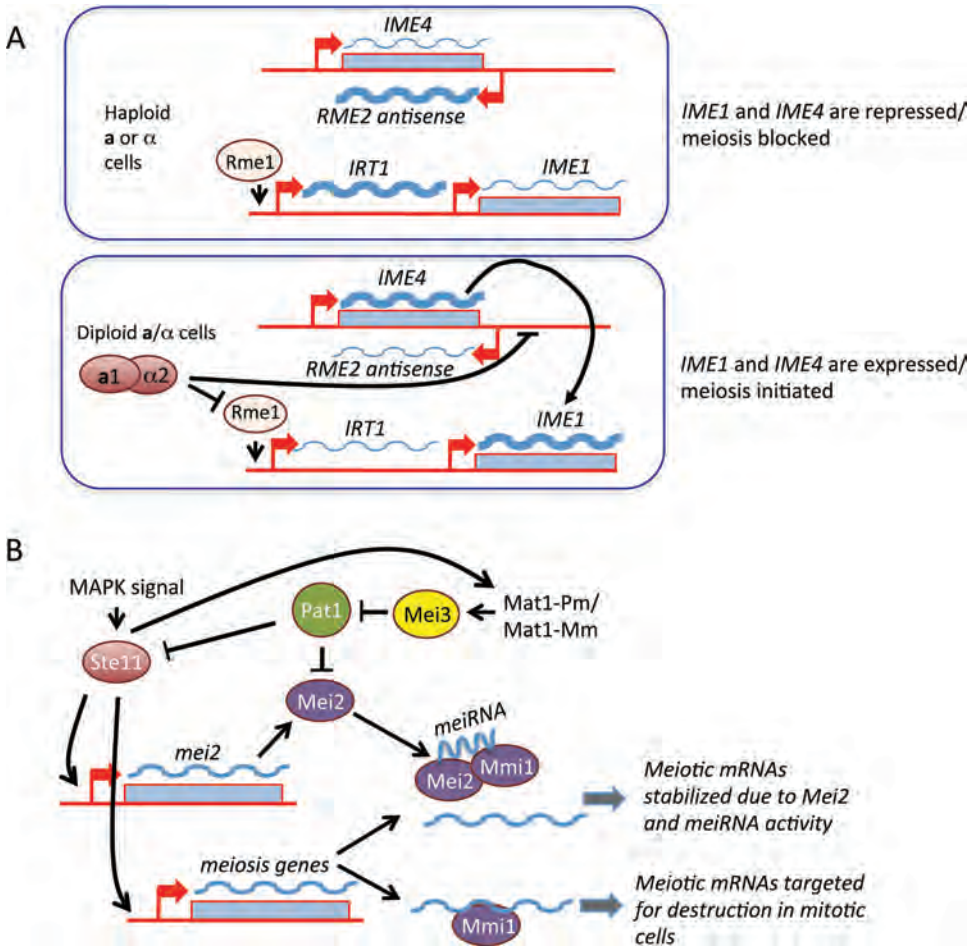


Figure 5 Regulation of meiosis in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. (**A**) In *S. cerevisiae*, two long noncoding RNAs, *IRT1* and *RME2*, regulate the expression of *IME1* and *IME4*, respectively, thereby controlling entry into meiosis. (**B**) In *S. pombe*, Mei2 and the long noncoding RNA meiRNA play a central role in meiotic regulation by suppressing Mmi1 and thereby stabilizing mRNAs necessary for entry into meiosis.

produced in an active form only in diploid P/M cells under starvation conditions. As in *S. cerevisiae*, waves of meiotic gene expression regulate progression through this developmental program (83).

Mating Type Switching in Yeasts

Both *S. cerevisiae* and *S. pombe* exhibit mating-type switching, allowing cells in a colony of one mating type to switch to the opposite mating type and then mate and produce sexual spores. Switching involves the copying of genetic information from a silent cassette to an active transcriptional locus. Notably, switching evolved independently in the lineages leading to *S. cerevisiae* and *S. pombe*, as discussed below.

In *S. cerevisiae*, cell type identity is defined by genes encoded at the *MAT* locus, with two additional silent cassettes present at the *HML*α and *HMR***a** loci (Fig. 6). Silent cassettes are kept inactive by flanking silencer sequences in tandem with trans-acting factors (e.g., the silent information regulator proteins, Sir1/2/3/4) to keep these DNA regions in a heterochromatic state (87). Mating-type switching occurs when the HO endonuclease makes a double-strand DNA break (DSB) at the *MAT* locus, and DNA from one of the silent cassettes is copied via a synthesis-dependent strand-annealing mechanism (87). The HO enzyme is only expressed in mother cells that have undergone cell division due to asymmetric localization of Ash1, a transcriptional inhibitor of *HO* expression, to the daughter cell (88–91). The HO protein evolved from homing endonucleases that replicate by a mechanism related to mating-type switching (92).

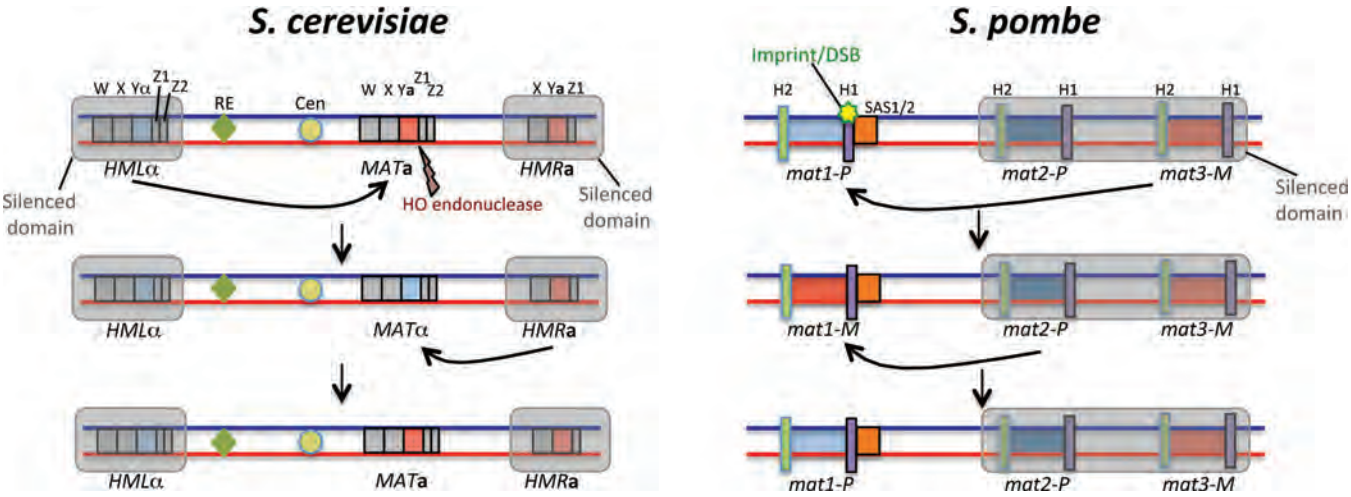


Figure 6 Mating type cassettes in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In both yeasts, mating type switching occurs by copying genetic information from a silent cassette into the transcriptionally active locus. In *S. cerevisiae*, silent cassettes are present at *HML*α and *HMR***a** and are copied into the active *MAT* locus. Recombination is activated by a DNA double-strand break introduced by the HO endonuclease at *MAT*. A recombination enhancer (RE) promotes recombination between *MAT***a** and *HML*α. In *S. pombe*, silent cassettes are present at *mat2-P* and *mat3-M* and are copied into the active *mat1* locus. Each cassette is flanked by homology regions (H1 and H2), and an imprinting event at H1 leads to recombinational repair of the damage using DNA from a silent cassette.

Mating-type switching exhibits donor preference, so that *MAT***a** strongly favors recombination with *HML*α, while *MAT*α favors recombination with *HMR***a** (93–96). Donor preference occurs because a recombination enhancer is present between *HML*α and the centromere (97, 98). In *MAT***a** cells, the recombination enhancer is active and, together with protein cofactors, promotes a conformational change in the chromosome structure so that the DSB at *MAT***a** is brought into close proximity with *HML*α (99, 100). The recombination enhancer is inactivated in *MAT*α cells by binding of Mcm1-α2, so that *MAT*α now preferentially recombines with *HMR***a**.

In *S. pombe*, pedigree analysis revealed that only one in four granddaughter cells undergoes a mating-type switch and that the sister of the switched cell is now also competent for mating-type switching (101, 102). To explain these findings it was proposed that a strand-specific imprinting event occurs at the *mat1* locus during DNA synthesis. DNA replication proceeds unidirectionally through the *mat1* locus due to a replication fork barrier at *RTS1*, and it was envisaged that imprinting occurs at *mat1* during lagging strand DNA synthesis (see Fig. 7). In the next round of DNA replication, leading-strand replication results in a transient DSB at the site of the imprint and initiates recombination with one of two silent cassettes, *mat2-P* or *mat3-M* (102–104). The nature of the imprint is unclear, although it could be an RNA primer left from lagging strand DNA synthesis that has been ligated to form a DNA-RNA-DNA hybrid (103, 105).

Similar to mating-type switching in *S. cerevisiae*, switching in *S. pombe* is biased so that the *mat1-P* cassette preferentially recombines with *mat3-M*, and *mat1-M* preferentially recombines with *mat2-P* (106). Recombination enhancers close to *mat2-P* and *mat3-M* contribute to these switching events (107). In addition, the *mat1-Mc* protein is important for directionality by promoting the expression of a truncated *swi2* transcript (108). The truncated Swi2 protein mediates differences in heterochromatin structure at *mat2-P* and *mat3-M*, thereby changing the bias of donor cassette choice (108).

Evolution of Mating-Type Switching

Mating-type switching arose independently in the lineages leading to *S. cerevisiae* and *S. pombe*, yet the mechanisms in both species involve three *MAT*-like loci (including two silenced loci), a DSB-initiated recombination event, a donor bias in cassette exchange, and a cell lineage tracking system (mediated by Ash1 in *S. cerevisiae* and genomic imprinting in *S. pombe*). In addition to promoting sexual reproduction, mating-type switching may provide a selective advantage by

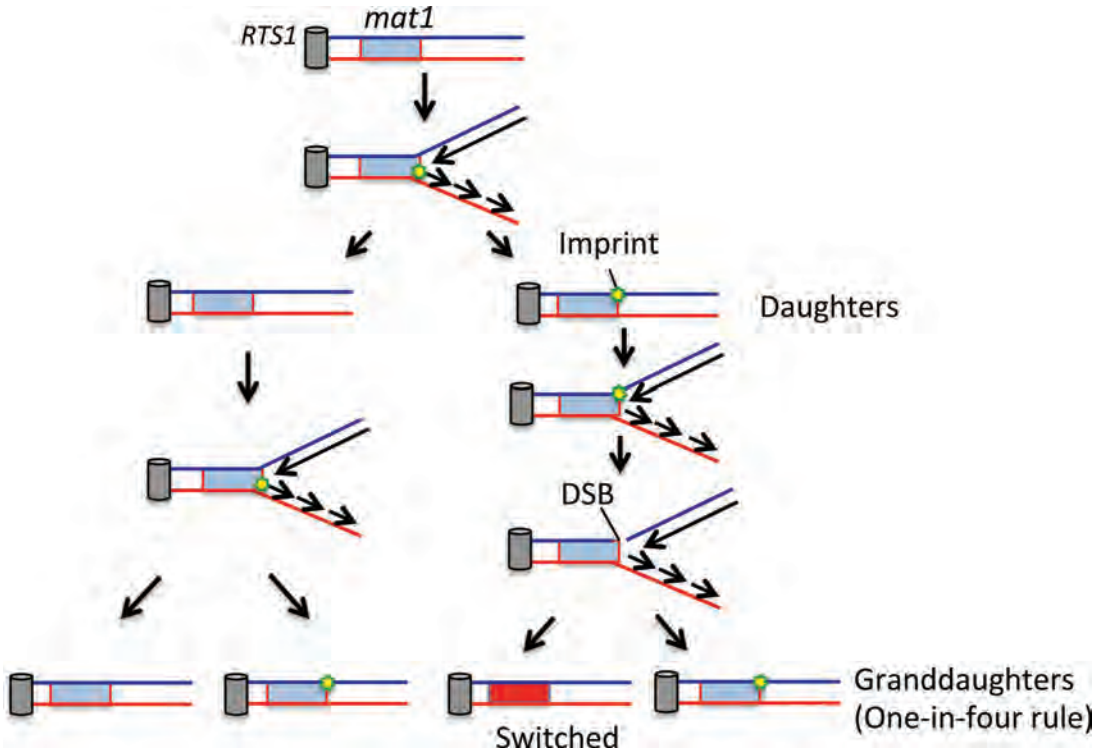


Figure 7 Mechanism of mating-type switching in *S. pombe*. During mating-type switching, a DNA imprint is first introduced during lagging strand DNA replication at the *mat1* locus. During the next round of DNA replication, the imprint is converted into a DNA double-strand break by leading strand synthesis. The DNA break initiates recombinational repair with one of the silent *mat* cassettes (*mat2-P* or *mat3-M*), resulting in switching of the cassette at the active locus.

allowing isolated cells to generate sexual spores that can subsequently survive nutrient-poor environments (109, 110). This could be important for yeasts such as *S. cerevisiae* and *S. pombe*, because unlike many other fungal species they are unable to make asexual spores by conidiation.

Recently, novel switching mechanisms were discovered in two methylotrophs, *Hansenula polymorpha* and *Pichia pastoris*, that shed light on how switching may have evolved in the ascomycetes (111, 112). In both species there are two linked *MAT*-like loci, one representing *MAT***a** and the other, *MAT*α. However, only one of these loci is actively expressed, whereas the other is silenced or expressed only at a reduced level. Mating-type switching occurs by recombination between inverted-repeat sequences and results in expression of the opposite mating type (Fig. 8A). Interestingly, these findings suggest that mating-type switching evolved in the ancestor to the lineage leading to the *Saccharomycetaceae* and the methylotrophs and was subsequently lost in the lineage leading to the *Candida* clade (Fig. 8B). The more primitive mating-type switching found in modern-day methylotrophs could have evolved into the three-*MAT* loci system present in *Saccharomycetaceae*, because this more efficient switching mechanism yields a higher spore yield (111). Conversely, loss of switching in some lineages could indicate that these species do not require frequent spore formation for either survival or dispersal (111).

SEXUAL REPRODUCTION IN *CANDIDA* SPECIES

Within the *Saccharomycotina* is a monophyletic group of species referred to as the *Candida* clade that includes significant human pathogens such as *C. albicans* (113–115). Historically, *Candida* referred to asexual or imperfect yeasts, but it is now known that many *Candida* species are competent for sexual or parasexual reproduction. The *Candida* clade can be further subdivided into haploid and diploid subclades, and several haploid *Candida* species are competent to undergo full sexual cycles (113). The best-studied haploid species is *Candida lusitaniae*, which undergoes meiosis despite

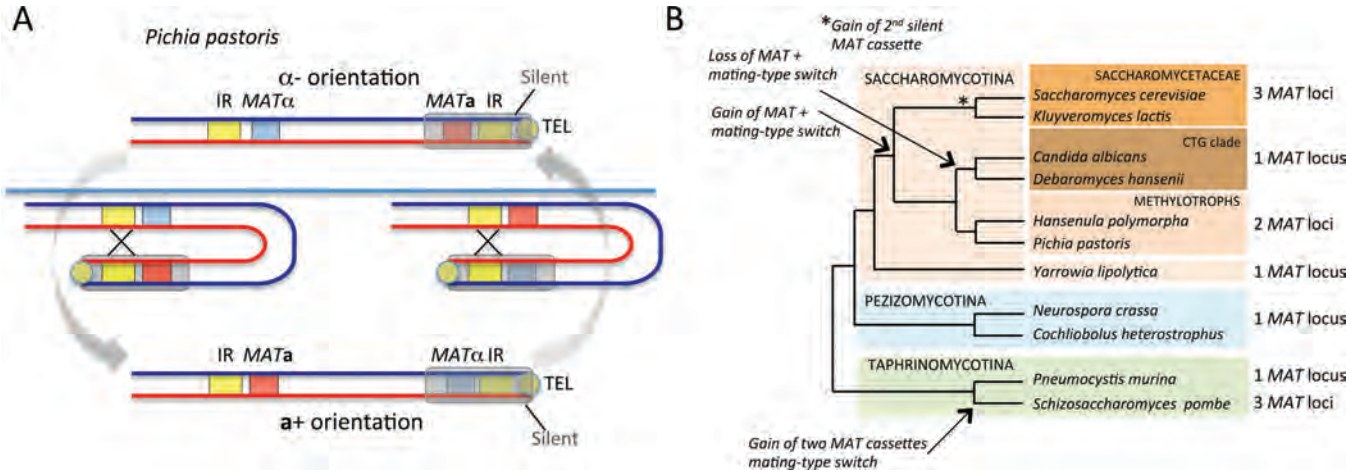


Figure 8 Evolution of mating type-switching mechanisms. (**A**) In *Pichia pastoris*, mating-type switching occurs by a flip-flop inversion mechanism. Inverted repeat (IR) regions flank a transcriptionally active *MAT* locus and a silenced *MAT* locus, the latter being located close to the telomere (TEL). Recombination events between IR regions lead to a change in mating type. (**B**) Model for how mating type switching evolved in the *Ascomycota*. Note that both *Hanenula polymorpha* and *P. pastoris* exhibit a similar inversion mechanism for mating-type switching. Adapted from reference 111.

lacking the meiotic recombinase Rad51, synaptonemal complex proteins, and the crossover interference pathway (see dyads formed by meiosis in Fig. 4) (113, 116). The MAPK transcription factor Ste12 is essential for both mating and meiosis in *C. lusitaniae* (117, 118). This resembles the scenario in *S. pombe*, where the same transcription factor drives both developmental programs. Other species in the haploid subclade include *Candida guilliermondii*, which exhibits heterothallic mating, as well as the homothallic (self-mating) species *Debaryomyces hansenii* and *Lodderomyces elongisporus* (113, 116).

Diploid *Candida* clade species include *C. albicans*, the most common cause of nosocomial fungal infections (119, 120). A mating-type-like (*MTL*) locus was identified in *C. albicans* and led to the demonstration that mating can take place between **a** and α cells, both *in vitro* and in a mammalian host (see image of mating zygote, Fig. 4) (121–123). Miller and Johnson showed that efficient mating requires cells to undergo a phenotypic switch from the default white state to the mating-competent opaque state (124). This regulatory mechanism has also been observed in two closely related *Candida* species (125–127). White-opaque switching involves a complex interplay between multiple transcriptional feedback loops, as well as regulation by chromatin modifications (for reviews see 128–131). In addition to regulating mating, the white-opaque switch influences many other traits, including filamentation, interactions with host cells, and virulence (132–135).

Why did the white-opaque switch evolve in a subset of *Candida* species? It is possible that the switch emerged to limit sexual reproduction to specific niches in the mammalian host (136). In support of this hypothesis, white and opaque cells exhibit different tissue trophisms (137, 138). Mating in the host could also facilitate the formation of specialized "sexual biofilms," because opaque **a** and α cells secrete pheromones that cause white cells of the opposite mating type to become adherent and form biofilms (139). Sexual biofilms stabilize pheromone gradients between opaque **a** and α cells, thereby stimulating conjugation between these cell types (139, 140).

Pheromone signaling between *C. albicans* opaque cells proceeds by a mechanism similar to that in *S. cerevisiae*, although some notable differences exist. For example, the *C. albicans* Ste5 (Cst5) scaffold coordinates MAPK signaling even though it lacks the von Willebrand A domain present in *S. cerevisiae* Ste5, which mediates binding to Ste7 (30, 141). Despite lacking this domain, *C. albicans* Ste5/Cst5 still binds to the MAPKKK, the MAPKK, and the MAPK to direct pheromone signaling (141). *C. albicans* also contains a Far1 ortholog, and in contrast to *S. cerevisiae*, loss of this scaffold blocks transcriptional responses to pheromone, as well as cell cycle arrest and polarized growth (142).

Some diploid *Candida* clade species, including *Candida parapsilosis* and its sister species *Candida metapsilosis* and *Candida orthopsilosis*, have not been observed to undergo sexual reproduction in the labora-

tory (143). *C. parapsilosis* isolates are all of the *MTL***a**/**a** mating type and contain a disrupting mutation in the *MTLa1* gene, suggesting that the *MTL* locus has degenerated and the ability to undergo sex has been lost (143). However, analysis of natural variation in *C. parapsilosis* isolates found evidence for interstrain recombination, suggestive of a population that has undergone sexual mating (144). Furthermore, a comparative analysis of *C. orthopsilosis* and *C. metapsilosis* genomes provides evidence for hybridization events due to mating between parental lineages (145, 146). Questions therefore remain concerning the extent to which various *Candida* species undergo sexual reproduction, both in the laboratory and in nature.

REWIRING CELL IDENTITY IN YEASTS

The ability to compare cell identity mechanisms across multiple *Saccharomycotina* species has revealed striking differences between species. Thus, whereas **a**-specific genes are constitutively expressed in *S. cerevisiae*, their expression in *C. albicans* requires the *MTL***a**-encoded factor **a**2, which is absent from *S. cerevisiae* (147, 148). Furthermore, *S. cerevisiae* represses **a**-specific genes in α cells by means of the α2 protein, whereas this mechanism does not operate in *C. albicans*. By comparative analysis of multiple *Saccharomycotina*, it is inferred that *C. albicans* has retained the ancestral mechanism for regulation of cell identity, whereas *S. cerevisiae* exhibits a derived circuit.

Examination of additional species has revealed a hybrid form of regulation in some *Kluyveromyces* species, with **a**-specific genes being both positively regulated by **a**2 and negatively regulated by α2 (149). This hybrid state was resolved by different routes during evolution; some species reverted to the ancestral regulatory circuit (e.g., *C. albicans*), whereas others, such as *S. cerevisiae*, retained only the new regulatory circuit (149). Hybrid circuits represent high potential states for evolution: they can be resolved by alternative routes without disruption to the overall circuit logic. The hybrid form of regulation could have provided a fitness advantage to the ancestor of *Kluyveromyces* and *Saccharomyces* species, because it would have arisen around the same time as the emergence of silent mating cassettes (149, 150). The ability to repress **a**-specific genes could have been advantageous in cases where leaky expression of **a**2 from silent cassettes would have inappropriately activated **a**-specific gene expression (149). These studies demonstrate how the comparative analysis of genetic circuits from multiple species can provide insights into the rewiring of transcriptional pathways during evolution.

PARASEXUAL REPRODUCTION IN *CANDIDA*

In most fungal species, a meiotic program is used to complete sexual reproduction. However, a conventional meiosis has not been observed in *C. albicans*, and instead, tetraploid cells (the products of diploid **a** and α mating) undergo a parasexual program of concerted chromosome loss (CCL) (151–153). Parasex is thought to involve chromosome nondisjunction events during mitotic divisions, and the products of parasex are therefore aneuploid cells that exhibit a wide variety of ploidies (151–153). A subset of progeny undergoes genetic recombination during the parasexual process, and the "meiosis-specific" recombinase Spo11 is implicated in this process (151).

Recent experiments demonstrate that haploid forms of *C. albicans* also can be generated by CCL (154). Haploid forms were originally thought to be inviable due to the presence of recessive lethal alleles in the diploid *C. albicans* genome. However, rare haploid forms were produced both *in vitro* and in a mammalian model of oral candidiasis. Haploid cells exhibit many of the attributes of diploid cells, including white-to-opaque switching and mating (154). However, although haploid cells represent an exciting breakthrough for genetic studies of *C. albicans*, they are currently limited in use due to their low fitness and tendency to spontaneously rediploidize (154).

HOMOTHALLIC MATING IN *CANDIDA*

As mentioned above, *Candida* clade species do not contain silent mating-type cassettes or undergo mating-type switching, yet several species are capable of homothallic mating. In *C. albicans*, **a** cells secrete both **a** and α pheromones, with α pheromone normally degraded by an aspartyl protease, Bar1, that is secreted only by **a** cells. However, in *bar1/bar1* mutants, **a** cells experience autocrine α pheromone signaling, resulting in activation of mating responses and productive unisexual **a**-**a** mating events (155). Same-sex **a**-**a** and α-α mating products (as well as conventional **a**-α products) are also observed in experiments in which **a** and α cells are mixed with one another (155). Additional experiments established that pheromone signaling alone is sufficient to induce same-sex mating in *C. albicans* (156). It is therefore possible that mating could be induced by pheromone-like peptides encountered in the mammalian host (156).

As noted above, there are also haploid *Candida* clade species that are homothallic, including *L. elongisporus* and *D. hansenii* (113). In *D. hansenii*, the two

MTL loci have become fused into one locus containing *MTLa1*, *MTLa2*, and *MTL2* genes (157). Presumably, the expression of these transcription factors enables the expression of both "**a**-specific" and "α-specific" genes in one cell to enable self-fertility. In contrast, all of the mating-type genes are missing from *L. elongisporus*, as well as the genes for **a**-factor and the receptor for **a**-factor (113). One possibility is that *L. elongisporus* uses autocrine α pheromone signaling to activate self-mating, similar to the mechanism described for *C. albicans*.

SEXUAL DEVELOPMENT IN FILAMENTOUS ASCOMYCETES

Sexual reproduction in filamentous ascomycetes (*Pezizomycotina*) is a more complex developmental process than that in unicellular yeasts (see Fig. 9), and requires self/nonself recognition between cells of opposite mating types, as well as between nuclei in a common cytoplasm (158). Most filamentous fungi are hermaphroditic, meaning an individual strain can behave as male or female. The female partner is a specialized hypha, the trichogyne, which develops from a protoperithecium formed at the beginning of the sexual phase, whereas the male partner is a conidium, or hyphal cell. Fertilization is accomplished when a male cell nucleus enters the trichogyne. Nuclei of opposite mating types do not fuse immediately after fertilization but divide in the female cytoplasm until a fruiting body (variously called a pseudothecium, perithecium, cleistothecium, apothecium, or gymnothecium) is erected (Fig. 4). A second recognition phase occurs when nuclei of opposite mating types discriminate self from nonself and enter specialized ascogenous hyphae. The apical cell of the dikaryotic ascogenous hypha typically differentiates into a hook-like crozier composed of several cells. Karyogamy ensues between two nuclei of opposite mating types compartmentalized in the penultimate cell; the antepenultimate and the apical cells contain one nucleus each, and these can subsequently fuse and reconstitute a dikaryotic cell that usually forms another crozier. The fused nucleus is in the only diploid cell in the life cycle and is diploid only briefly before the cell differentiates into a meiocyte and meiosis occurs. The meiocyte subsequently differentiates into the ascus mother cell. In filamentous ascomycetes, a mitosis usually follows meiosis, and the mature ascus

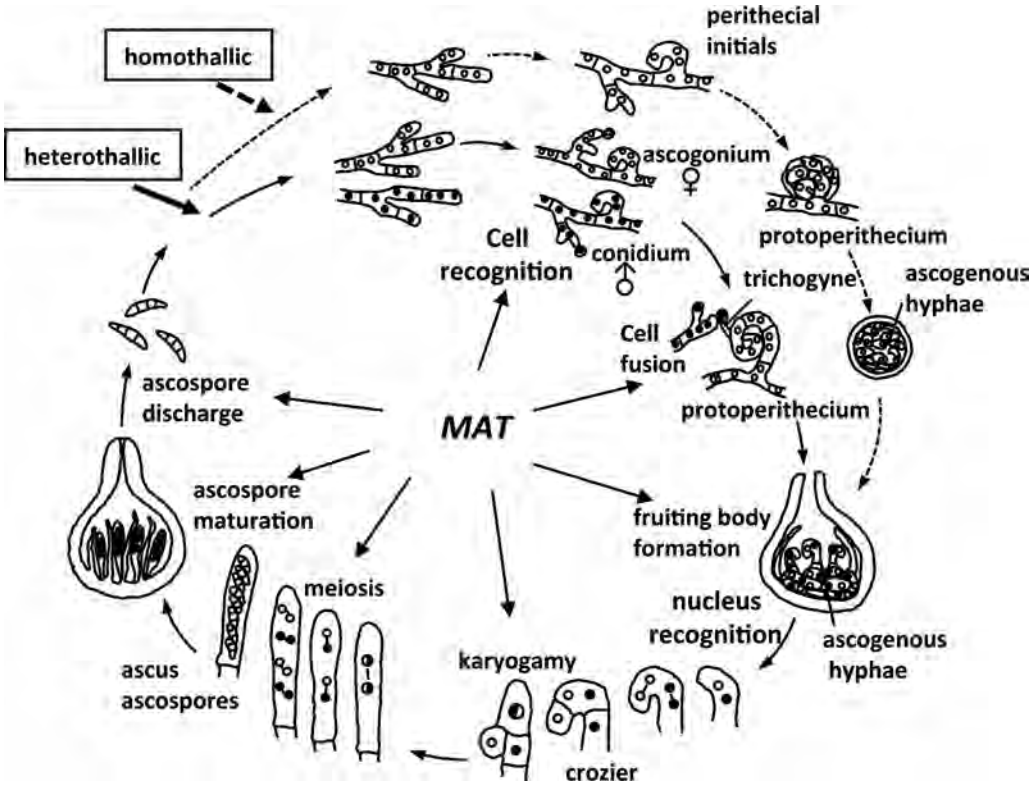


Figure 9 Generalized life cycle of filamentous *Pezizomycotina*. *MAT* impacts all stages indicated (see text). Heterothallic species, inner ring (solid); homothallic species, outer ring (dashed).

therefore contains eight spores (four pairs of twins), twice the number typical in yeasts (see *Cochliobolus heterostrophus* in Fig. 4).

In contrast to filamentous ascomycetes, unicellular yeasts do not bear reproductive spores inside developmentally complex fruiting bodies. The differentiation events within the female organs also constitute essential differences with ascomycete yeasts. In both filamentous and unicellular species, the mating-type proteins control fertilization and meiosis. However, in filamentous fungi, they also control internuclear recognition and the formation of dikaryotic hyphae (159). Ultimately, this complex cycle yields genetically variable progeny from a single fertilization event. Cytologically, the program of ascus development is similar in heterothallic and homothallic species (159–161), but the early cell-cell recognition events differ (Fig. 9).

Heterothallic Mating-Type Loci in *Pezizomycotina*

As for many ascomycetous yeasts, the mating-type locus in *Pezizomycotina* is a single *MAT* locus consisting of genes encoding key transcription factors (158, 159, 162, 163). *MAT* nomenclature is not uniform, in part because these loci were initially discovered genetically (164, 165) or sorted into mating types according to mating interactions before the molecular nature of the corresponding genes was known. Furthermore, nomenclature rules tend to be species-specific (as exemplified by the *S. cerevisiae* and *S. pombe MAT* loci described earlier). Upon molecular characterization (164–167) of *MAT*-encoded genes, Turgeon and Yoder (168) proposed that the locus be called *MAT1* and the alternate idiomorphs (169, 170) be called *MAT1-1* and *MAT1-2*. For ease of cross-species comparisons, it was suggested that these designations should be adopted as new heterothallic *MAT* loci were identified. The genes encoded at *MAT1-1* would be *MAT1-1-1*, *MAT1-1-2*, etc., and those encoded at *MAT1-2* would be *MAT1-2-1*, *MAT1-2-2*, etc., as needed. Fungi for which there was already a rich mating-type literature, such as *N. crassa* and *P. anserina*, would continue to be referred to by their original terminology (171, 172).

The *MAT1-2* idiomorph of most *Pezizomycotina* carries a single gene, *MAT1-2-1*, encoding a high mobility group (HMG) protein (173), whereas the *MAT1-1* idiomorph harbors a gene, *MAT1-1-1*, encoding an α1 box-containing protein (174) and may or may not harbor additional genes (Fig. 10) (159, 164, 172, 175–180). Notably, both *MAT* idiomorphs in an early diverging fungal lineage, the *Zygomycotina*, encode HMG-type proteins (181). *MAT*-like genes encoding HMG-type proteins also have been identified in the most basal class of fungi, the *Microsporidia* (182, 183). In tracing *MAT* gene evolutionary history, Martin et al. (184) determined that the α1 protein is a member of the HMG protein family, and thus the apparently unrelated *MAT1-2-1* and *MAT1-1-1* genes both encode HMG proteins (184). This discovery, together with the evidence that HMG proteins are present at the *MAT* locus in both mating types in early diverging lineages, supports the hypothesis that the ancestral fungal *MAT* alleles encoded HMG protein transcription factors that subsequently diverged (Fig. 11).

In the *Sordariomycetes*, *MAT1-1-1* encodes the α1 box (HMG) protein, *MAT1-1-2* encodes a protein with an HPG (also termed PPF) domain, and *MAT1-1-3* encodes another HMG-type protein (185, 186) (Fig. 10). Note that there are many genes that encode HMG-type proteins in fungal genomes (187). Deletion analysis of these genes in *P. anserina* demonstrated that 11 out of 12 HMG proteins are involved in regulation of the sexual cycle and highlighted their central importance in this developmental program. Several comprehensive reviews are available that describe the discovery, structural features, and function of *MAT* loci in *Pezizomycotina* (158, 159, 163, 169, 188–191).

Homothallic Mating in *Pezizomycotina*

A number of mechanisms allow single individuals to undergo sexual reproduction, including primary homothallism, pseudohomothallism, homothallism arising from mating-type switching (either bidirectional or unidirectional), and unisexual reproduction. The reader is referred to an excellent overview of these reproductive mechanisms by Wilson et al. (192).

Primary homothallism

In homothallic *Pezizomycotina*, a single individual can complete the sexual cycle because each nucleus in a genetically homogeneous strain carries both *MAT* genes. In an early seminal publication, Metzenberg and Glass (169) proposed that "homothallic species may be functionally heterothallic at the level of nuclei in the ascogenous hyphae" perhaps by an epigenetic mechanism, such as silencing of one of the two mating types. Other studies have proposed the same mechanism (193–195). We still do not have definitive proof of such a mechanism, but the net result would be as for yeasts that switch; functional heterothallism is achieved, and a diploid nucleus is formed from haploid nuclei.

Homothallic *Pezizomycotina MAT* loci can be very diverse compared to heterothallic *MAT* loci, which are generally conserved both within and across species

(159, 196–199). All heterothallic *Cochliobolus* species, for example, have the same *MAT* topology (197). In contrast, homothallic species carry *MAT1-1* and *MAT1-2* in a single nucleus but with strikingly different rearrangements and polyphyletic evolutionary origins (197). In two species, *Cochliobolus luttrellii* and *Cochliobolus homomorphus*, the *MAT* genes have become fused into a single open reading frame, and the corresponding proteins are truncated at the fusion junction. In two other examples, *MAT1-1* and *MAT1-2* are not fused. In *Cochliobolus kusanoi*, the organization is an inverted *MAT1-1-1* gene linked to a hybrid gene containing a truncated *MAT-1-1-1* fused to the full *MAT1-2-1* sequence. Fragments of genes known to be on the flanks of heterothallic relatives are interspersed, suggestive of accompanying, or additional, recombination events. In *Cochliobolus cymbopogonis*, homologs of both of the heterothallic idiomorphs are present but are not closely linked.

Both *MAT1-1* and *MAT1-2* are found in the same genome in members of other taxonomic groups. These include the *Sordariomycetes Fusarium graminearum* (196), *Sordaria macrospora* (200), and *Chaetomium globosum* (159); the *Eurotiomycete Aspergillus nidulans* (177, 201–203); and the *Leotiomycete Sclerotinia sclerotiorum* (180, 204). The latter carries both *MAT* idiomorphs fused end to end (180). The *MAT1-1* region has *MAT1-1-1* and *MAT1-1-5* genes, while the *MAT1-2* region harbors *MAT1-2-1* and *MAT1-2-4* genes, as it does in the close heterothallic relative *Botrytis cinerea* (Fig. 10). Two versions of the *MAT* region that differ by a 3.6-kb inversion are found in selfing populations. The inversion is likely promoted by a 250-bp repeat within the *MAT1-1-1* and *MAT1-2-1* genes, which changes orientation of the genes, truncates one gene, and is correlated with altered gene expression.

Heterothallic *B. cinerea* itself has an unusual *MAT* structure, with a truncated copy of *MAT1-1-1* in the *MAT1-2* mating region and a truncated copy of *MAT1-2-1* in the *MAT1-1* region. Comparisons of heterothallic *B. cinerea* with homothallic *S. sclerotiorum* suggest that inversions and deletions in the latter *MAT* region led to the present-day structures in heterothallic *B. cinerea*. Some *B. cinerea* isolates are also homothallic (205, 206) and yet can still outcross to *MAT1-1* and *MAT1-2* strains. Analysis of several homothallic strains revealed that they do not carry both *MAT* regions and therefore are analogous to species that exhibit unisexual mating (see section below).

As noted, several other species such as *S. sclerotiorum*, *F. graminearum*, and *A. nidulans* (180, 207–209) can also both self- and outcross, i.e., exhibit both self-compatible and self-incompatible behavior, although this is relatively rare (210–213). Often, homothallic strains do not produce asexual spores (e.g., *Neurospora* species), which may underlie the inability to outcross.

Bidirectional mating-type switching

As discussed above, several yeast species contain silent mating-type cassettes that can be inserted into the active *MAT* locus, leading to switching of cell type, and thus appear homothallic. Silent mating-type cassettes have not been reported in *Pezizomycotina* genomes, however, and there is no evidence that these species can undergo switching by this mechanism.

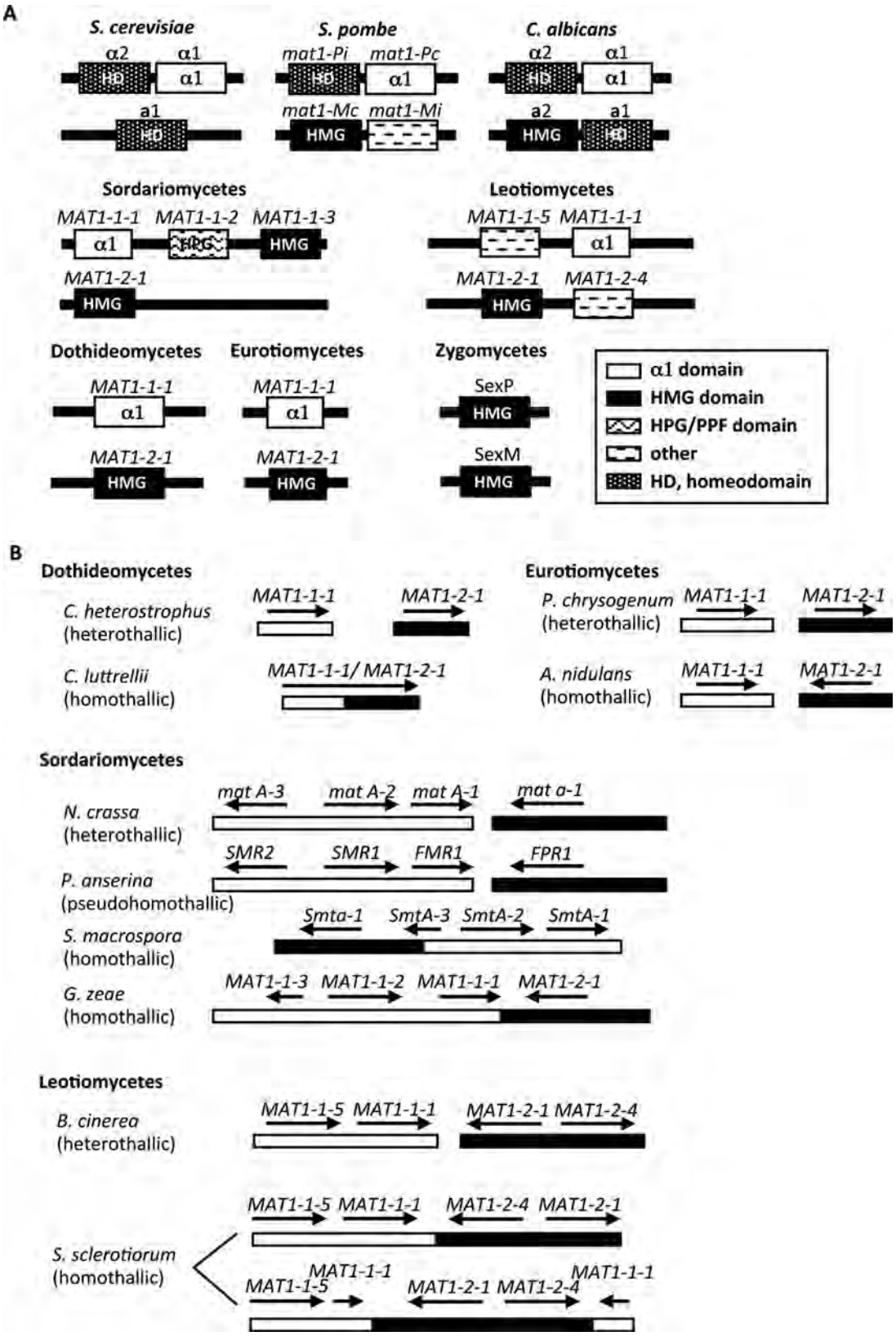
Unidirectional mating-type switching

An unusual case of unidirectional switching has been described for the *Sordariomycete Chromocrea spinulosa* (*Hypocrea spinulosa*). This species is found as two cell types that are capable of mating with each other, but only one of these can undergo self-mating (214). When the two cell types are mated with one another or when self-compatible progeny undergo sexual reproduction, the resulting progeny segregate 1:1 self-compatible: self-incompatible. Molecular characterization reveals that self-incompatible strains are all *MAT1-1* and have the three-gene structure typical of the *Sordariomycete MAT* locus (215). In contrast, self-compatible strains have *MAT1-1* plus a second copy of *MAT1-1* linked to a *MAT1-2-1* gene. The presence of a repeated sequence hints at an intramolecular, unidirectional switching mechanism whereby the *MAT1-2-1* gene is eliminated, resulting in a strain with *MAT1-1* that can only undergo outcrossing.

Recent studies of unidirectional mating-type switching in species of *Ceratocystidaceae* have shown that self-fertile isolates of *Ceratocystis fimbriata* and *Ceratocystis albifundus* have three genes at the *MAT1* locus (*MAT1-1-1*, *MAT1-1-2*, and *MAT1-2-1*) (192). Ascospore progeny are either self-fertile or self-sterile as for *C. spinulosa*, and it has been shown that elimination of the *MAT1-2-1* gene is responsible for the self-sterile phenotype (216, 217). The mechanism by which this occurs is unknown but could be promoted by homologous recombination within repeats that flank the *MAT1-2-1* gene, similar to the mechanism envisaged for *C. spinulosa*.

Pseudohomothallism (secondary homothallism)

Pseudohomothallism refers to species that are self-fertile because nuclei representing each mating type are separately maintained in parental cells and progeny.


A
S. cerevisiae
α2
α1
HD
α1
a1
HD
S. pombe
mat1-Pi
mat1-Pc
HD
α1
mat1-Mc
mat1-Mi
HMG
C. albicans
α2
α1
HD
α1
a2
a1
HMG
HD
Sordariomycetes
MAT1-1-1
MAT1-1-2
MAT1-1-3
α1
HPG
HMG
MAT1-2-1
HMG
Leotiomycetes
MAT1-1-5
MAT1-1-1
α1
MAT1-2-1
MAT1-2-4
HMG
Dothideomycetes
MAT1-1-1
α1
MAT1-2-1
HMG
Eurotiomycetes
MAT1-1-1
α1
MAT1-2-1
HMG
Zygomycetes
SexP
HMG
SexM
HMG
α1 domain
HMG domain
HPG/PPF domain
other
HD, homeodomain
B
Dothideomycetes
C. heterostrophus
(heterothallic)
MAT1-1-1
MAT1-2-1
C. luttrellii
(homothallic)
MAT1-1-1/ MAT1-2-1
Eurotiomycetes
P. chrysogenum
(heterothallic)
MAT1-1-1
MAT1-2-1
A. nidulans
(homothallic)
MAT1-1-1
MAT1-2-1
Sordariomycetes
N. crassa
(heterothallic)
mat A-3
mat A-2
mat A-1
mat a-1
P. anserina
(pseudohomothallic)
SMR2
SMR1
FMR1
FPR1
S. macrospora
(homothallic)
Smta-1
SmtA-3
SmtA-2
SmtA-1
G. zeae
(homothallic)
MAT1-1-3
MAT1-1-2
MAT1-1-1
MAT1-2-1
Leotiomycetes
B. cinerea
(heterothallic)
MAT1-1-5
MAT1-1-1
MAT1-2-1
MAT1-2-4
S. sclerotiorum
(homothallic)
MAT1-1-5
MAT1-1-1
MAT1-2-4
MAT1-2-1
MAT1-1-5
MAT1-1-1
MAT1-2-1
MAT1-2-4
MAT1-1-1

P. anserina and *Neurospora tetrasperma* have served as models for this lifestyle and can both self- and outcross (218–221). Both species can resolve naturally into homokaryotic strains differing in mating type by loss of one of the two nuclei or by inefficient recombination mechanisms.

How do pseudohomothallic fungi generate dikaryotic cells during sexual reproduction? Both *P. anserina* and *N. tetrasperma* undergo defined nuclear divisions during meiosis that result in two nonsister nuclei being packaged together into ascospores. The precise meiotic divisions are different in the two species (for a recent review see 222), but both species rely on limiting recombination around the mating-type locus. In *N. tetrasperma*, the mating region is on a 9.4-Mb chromosome, with ~7 Mb of this chromosome repressed for recombination (~1,500 genes). There are several large inversions which may account for recombination suppression (223). The *P. anserina mat* region extends for ~0.8 Mb and is similarly suppressed for recombination over this length. In this species, however, it is unknown how recombination is prevented in the suppressed region. For both *N. tetrasperma* and *P. anserina* the regulation of recombination events close to *mat* are necessary for the correct packaging of dikaryons during meiosis (222).

Samils et al. (224) recently tracked differential gene expression in homokaryotic *N. tetrasperma* strains and in heterothallic *N. crassa* strains containing different mating types. These analyses revealed a mating-type bias and preferential enrichment for differentially expressed genes on the *mat* chromosome. Thus, *mat A* target genes were associated with sexual and female development, while *mat a* target genes were associated with vegetative and male development, indicative of sexual dimorphism. In subsequent work, analysis of *mat*-linked single nucleotide polymorphisms in three different heterokaryons examined the ratio of *mat A* to *mat a* nuclei during development; *mat A* nuclei were present at higher levels than *mat a* nuclei during mycelial growth (87% *mat A*), whereas during sexual development the nuclear ratio became more even (40 to 57% *mat A*) and there was an expression bias toward *mat a*-linked genes. In short, both the nuclear ratio and biases in *mat A/mat a* target gene expression may contribute to the fitness of heterokaryons throughout the life cycle (225).

Unisexual mating

In primary and secondary homothallism, fungal species either display the presence of both mating-type genes in a single genome or have two opposite mating-type nuclei in a single cell, respectively. However, some species are capable of unisexual mating (selfing), in which all nuclei contain a single mating-type idiomorph only. Unisexual mating was discovered in homothallic *Neurospora* species (198, 226, 227) and was recently described for *Huntiella moniliformis*, which carries the *MAT1-2-1* gene, in contrast to *Neurospora africana*, which carries the *MAT1-1-1* gene (228). As discussed earlier, unisexual mating is also observed in some *Candida* clade species, as well as in the basidiomycetes (229, 230).

Nonbipolar homothallism

Mating behavior by the sordariomycete *Glomerella cingulata*, first described in 1914, has remained "atypical" since the initial publication (231–233). Two types of self-fertile strains (plus and minus) exist, and these strains can also outcross. Cisar and TeBeest (233) came to the conclusion, after exhaustive crossing experiments, that *G. cingulata* does not have a simple bipolar mating system but rather involves a single locus with multiple idiomorphs. To our knowledge there has been no follow-up work with this species.

Heterologous Expression of *MAT* Genes

The cross-species expression of *MAT* genes has been examined in only a handful of fungal species (178, 234–237). It has been demonstrated, for example, that self-fertility does not transfer to heterothallic *N. crassa* from a closely related homothallic *Neurospora* species (226). In contrast, manipulation of *MAT* loci in heterothallic and homothallic *Cochliobolus* species has been informative regarding *MAT* protein contributions to sexual development. These studies have established that heterothallism preceded homothallism in this genus (197), and this evolutionary trend has since been confirmed for a number of additional fungal groups (198). Yun et al. (197) examined whether the transfer of a ho-

Figure 10 Organization of the *MAT* locus in model *Ascomycetes* (and *Zygomycetes*). (**A**) Black lines, idiomorphs; rectangles, MAT proteins with signature domains. Domain type is indicated in the large box on the right. Note the diversity of *MAT* locus organization but recurrent types of protein carried. (**B**)*MAT* organization in heterothallic and homothallic representatives of each large class of fungi. White boxes, *MAT1-1* idiomorph; black boxes, *MAT1-2* idiomorph. Genes and their direction of transcription are noted. See text for details.

mothallic *MAT* region into a heterothallic *mat*-deletion strain, or vice versa, could alter the reproductive lifestyle. These studies demonstrated that:

1. A *mat*-deletion strain of heterothallic *C. heterostrophus* could be made functionally homothallic upon transfer of the fused *MAT* gene from homothallic *C. luttrellii*. Transgenic strains could self and produce both fruiting structures (pseudothecia) and viable asci and ascospores. Thus, the homothallic *MAT* gene alone is able to mediate the switch from heterothallism to homothallism (197). This is in direct contrast to results obtained when both *C. heterostrophus* heterothallic *MAT* genes are added to the same *C. heterostrophus* *mat*-deletion strain; pseudothecia form, but these are not fertile (no asci or ascospores) (238).
2. The fused *MAT* locus from a more distantly related homothallic species, *C. homomorphus*, was able to initiate the sexual developmental process in the same *mat*-deletion strain of heterothallic *C. heterostrophus*, but pseudothecia that formed were barren (no asci). This experiment establishes that structural differences between *C. luttrellii* and

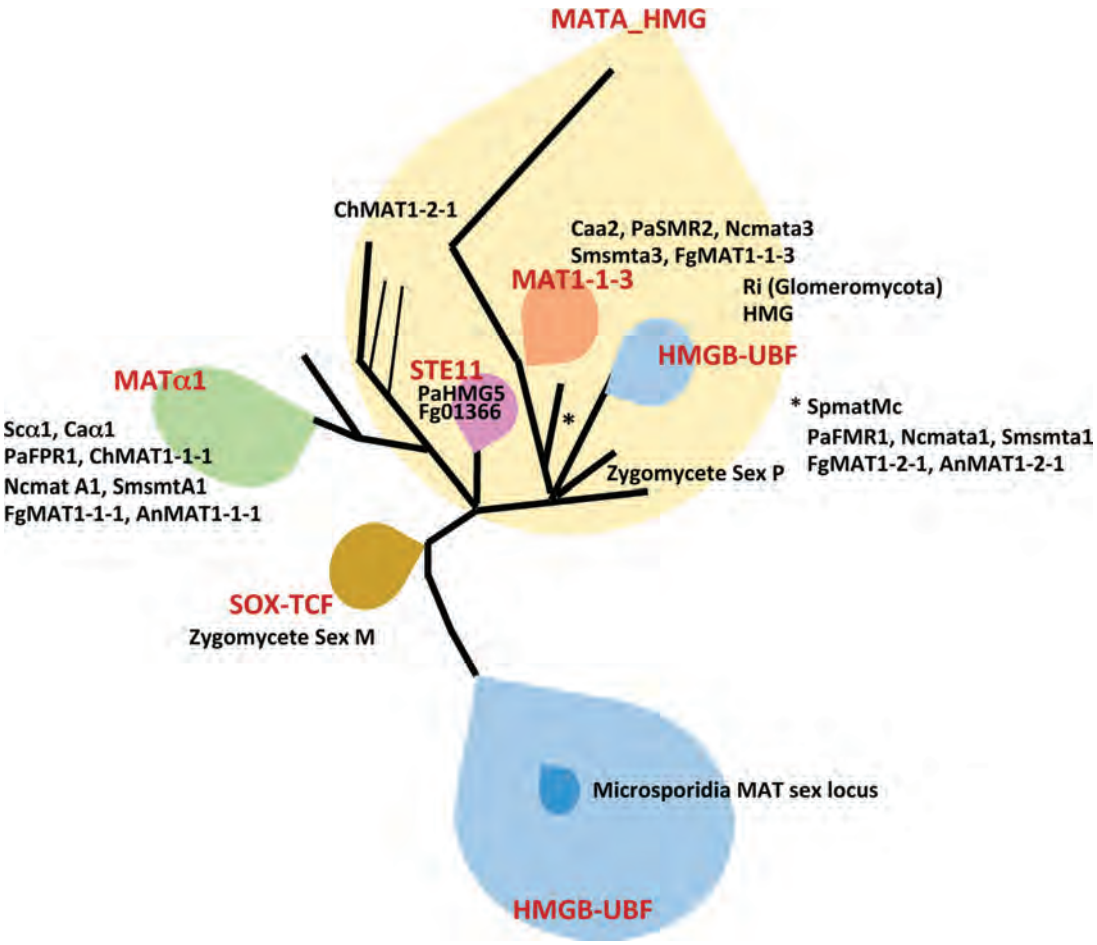


Figure 11 Phylogenetic relationships among members of the HMG-box superfamily. Adapted from Fig. 3 of reference 184 and Fig. 2 of reference 187. Note that the alpha 1 domain (α1) is an HMG protein. Colors: MATα_HMG, green; MATA_HMG, cream; SOX-TCF, brown; HMGB-UBF, light blue; MAT1-1-3 in MATA, orange; STE11 in MATA, purple. Other labels: Microsporidia MAT sex locus in HMGB-UBF (dark blue), *Phycomyces blakesleeanus* (Zygomycota) sexM and sexP, *Rhizophagus irregularis* (Glomeromycota) HMG proteins in MATA_HMG group. Abbreviations: An, *Aspergillus nidulans*; Ca, *Candida albicans*; Ch, *Cochliobolus heterostrophus*; Fg, *Fusarium graminearum*; Pa, *Podospora anserina*; Nc, *Neurospora crassa*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Sm, *Sordaria macrospora*.

C. homomorphus MAT genes hold the key to homothallic fertility.

3. A *mat*-deletion strain of homothallic *C. luttrellii* was made heterothallic by expression of *C. heterostrophus MAT1-1-1* and *MAT1-2-1*; the two transgenic strains, differing in heterothallic mating type, could mate and produce both pseudothecia and viable ascospores, indicating that *MAT* alone is able to induce the switch from homothallism to heterothallism (238). Interestingly, homothallic *C. luttrellii MAT*-deletion strains carrying only a single heterologous *MAT* gene from heterothallic *C. heterostrophus* were also capable of producing fertile pseudothecia. These were much smaller in size than those produced by an outcross and were only about 10% as fertile (but asci contained full tetrads) and can be considered unisexual mating events, as described above.

The contributions of *MAT* genes to mating also have been examined using the homothallic *Dothideomycete*, *Didymella zeae-maydis*. This species contains linked *MAT1-1-1* and *MAT1-2-1* genes (207). Strains deleted for *MAT1-2-1* were completely sterile when selfed, suggesting that the function of the MAT1-2-1 HMG protein is essential for mating. In contrast, selfing of strains deleted for *MAT1-1-1* produced some ascospores, although the fertility was much lower than in wild type self-mating. This suggests that the function of MAT1-1-1 (α1 domain) might be redundant with one or more of the other HMG proteins in the genome (187, 239). The ability of a strain with only a single *MAT* gene to self is reminiscent of the finding that homothallic *C. luttrellii mat*-deletion strains can also self if carrying a single *MAT* gene from heterothallic *C. heterostrophus* and is yet another example of unisexual mating.

The Search for Sex in "Asexual" Filamentous Ascomycetes

Sharon et al. (240) first demonstrated that fungi for which there was no evidence of sexual reproduction (apparently asexual species) carried mating-type genes. Both mating types of *Bipolaris sacchari*, an asexual relative of sexual *Cochliobolus*, could be found in *B. sacchari* populations. Furthermore, heterologous expression of *B. sacchari MAT* genes in a *mat*-deleted strain of heterothallic *C. heterostrophus* demonstrated that the *B. sacchari MAT* genes were fully functional. The reverse heterologous expression experiment (expression of *C. heterostrophus MAT1-1* and *MAT1-2* in *B. sacchari*) did not change the asexual phenotype. These data, together with phylogenic evidence, support the argument that asexual fungi arise from sexual ones and that defects in mating in asexual species could be attributable to mutations in any one of the suite of genes in the mating pathway.

In the human pathogen *A. fumigatus*, considerable effort was required before successful mating was observed in this species (241). Losada et al. (242) capitalized on this finding, making a highly fertile isogenic pair of strains that mate in a heterothallic manner, and showed that mating type is not associated with virulence. Swilaiman et al. recently demonstrated the same phenomenon for the human pathogen *Aspergillus lentulus* (243).

Mating-type genes have also been identified in two supposedly asexual dicot phytopathogens, *Fusarium oxysporum* (196) and *Alternaria alternata* (244). The *F. oxysporum* genes were shown to be expressed and highly similar to *MAT* genes in sexual *Fusarium* species. The *Alternaria* genes were functional in *mat*-deleted strains of *C. heterostrophus*, but under no condition could *Alternaria* strains of different mating types be shown to mate. More recently, population genetic analysis has provided support for cryptic sex in *A. alternata* populations, which could complicate eradication of this phytopathogen (245).

A debate surrounds the issue of how asexual lineages can persist without an invigorating sexual cycle (246, 247), with one idea being that there is elevated intragenomic diversity in asexual genomes. Notably, genome mining of the asexual mycorrhizal fungus *Rhizophagus irregularis* (a member of the *Glomeromycota*) identified genes for HMG proteins as found in all ascomycete lineages, as well as in the early diverging *Zygomycete* and *Microsporidial* fungi (248). Riley et al. (249) examined three isolates and extracted 76 HMG homologs, none of which was flanked by genes known to be on *MAT* flanks in other fungi. Intriguingly, a 9-kb-long region containing four tandem-repeated MATA-HMGs (Fig. 11) was identified which supports the notion that frequent gene duplications generate intragenomic diversity, which may save supposedly asexual *Glomeromycota* from extinction. Alternatively, cryptic sexual cycles may yet be discovered in many asexual fungal species.

MATING SIGNALING IN FILAMENTOUS ASCOMYCETES

MAT Gene Functions

In heterothallic ascomycetes, the *MAT* genes are required for recognition of strains of opposite mating type, as

well as for recognition of nuclei of opposite mating-type, fertilization, formation of the biparental ascogenous hyphae, and meiosis (see Fig. 9). *MAT* genes have been determined to play important roles in many homothallic species as well. For example, in homothallic *F. graminearum*, studies indicate that both *MAT* genes are required for production of fertile perithecia (185). In contrast, in *S. macrospora*, only one of the three *MAT1-1* genes (*SmtA-2/MAT1-1-2*) was found to be essential for sexual reproduction (250). An intriguing report on *P. anserina* (251) documented that mutations in the same gene (*SMR1/MAT1-1-2*) led to barren perithecia. Fifteen suppressor mutations of this phenotype were found; all were mutations in *MAT* genes, and all rendered mutants weakly self-fertile. This suggests that regulation of self-compatible/incompatible mating targets is determined by specific sequences in *MAT* itself.

Pheromones and Receptors

Genes encoding pheromones and their cognate receptors are essential for sexual development of heterothallic *Pezizomycotina* (252–254). In contrast, the roles of pheromones/pheromone receptors are less obvious in some homothallic species because mate recognition and attraction may be unnecessary (255). For example, although *F. graminearum* encodes genes for pheromones and receptors, neither the pheromones nor their cognate receptors are required for sexual development (256). Homothallic *S. macrospora* encodes, but does not strictly need, pheromones for mating (mutants exhibit reduced numbers of pseudothecia), but it does require products of the corresponding receptor genes for sexual development (250, 257). For *A. nidulans*, expression of the gene encoding the α pheromone precursor and the two receptor genes is increased during sexual development; however, expression of these genes is not under *MAT* control (203). For homothallic *Neurospora* species, one or more pheromone receptor genes are important for sexual development (258, 259).

Transcriptional Analysis of Mating

The genes regulated by *MAT* in filamentous ascomycetes have been described for several species, including *S. macrospora* (250, 257, 260), *F. graminearum* (261–264), *Aspergillus* species (265), *N. crassa* (224, 266, 267), and *P. anserina* (268). The studies with *N. crassa* demonstrated that there is rapid evolution of sex-associated genes and differential expression associated with mating type. The *P. anserina* study demonstrated that even conserved target genes could have different transcriptional profiles when multiple species are compared.

A recent transcriptional study of sexual development in *F. graminearum* by Kim et al. (185) brings the contributions of *MAT* genes and signaling elements in a homothallic species into sharper focus. It also highlights a common theme in this article: sexual reproductive pathways are plastic, and a component's function in one species is often repurposed in another. Previous work demonstrated that all four *MAT* genes are essential for sexual development (254, 269–271). Kim et al. (185) uncovered 1,245 differentially expressed genes by comparing different *MAT* mutants or comparing mutants with wild type strains. They identified 50 transcription factor-encoding genes; those with a specific and essential function in sexual development were enriched only among downregulated genes. Deletion of 106 of the differentially expressed genes pinpointed 25 genes specifically required for sexual development, with most regulated by both *MAT1-1* and *MAT1-2*. Among the sex-specific transcription factors is an ortholog (FGSG_01366) of PaHMG5, an upstream regulator of the *P. anserina MAT* genes *FMR1* and *FPR1* (187) and the pheromone/receptor system. In *F. graminearum*, however, FGSG_01366 appears to be a direct downstream target of *MAT*.

Sexual and asexual development in filamentous ascomycetes is also known to involve velvet complex regulation (272) and G protein signaling (273, 274). Mutants deleted for *VelB*, encoding a velvet complex component, or *GPA1*, encoding the α subunit of the tripartite G protein, are self-sterile, similar to *mat*-deletion strains (275, 276). Of the four *F. graminearum MAT* genes, three are downregulated in the *velB* mutant, while only one is downregulated in the *GPA1* mutant. More than half of the genes downregulated in the *mat*-deletion strains overlapped with genes downregulated in *velB*-deletion strains, while less than 20% overlapped with genes downregulated in the *gpa1*-deletion strain (250).

At this point, models of *MAT* circuitry in *Pezizomycotina* have largely relied on transcriptional profiling data, and few are supported by comprehensive functional analyses. However, robust genome-wide techniques, such as ChIP-seq, are beginning to shed light on transcriptional programs. One example is a study of *Penicillium chrysogenum* that reports direct binding targets of MAT1-1-1 and provides evidence for a conserved MAT1-1-1 (α1) DNA-binding motif (277). Known targets such as *ppg1*, the homolog of the *S. cerevisiae* gene α-factor pheromone-producing gene, and *pre1*, the homolog of *ScSTE3*, were confirmed in this study. Moreover, genes were identified that support the notion that *MAT* pathway components can be repurposed, as described in the following section.

ADDITIONAL ROLES FOR SEXUAL SIGNALING IN THE *ASCOMYCETES*

Recent studies have revealed that the fungal sexual machinery has been repurposed for alternative functions in some *Ascomycetes*. A prime example is provided by *Fusarium oxysporum*, a plant pathogen that can sense and grow toward chemicals produced by its tomato host. Experiments by Turrà et al. demonstrated that chemotrophic growth was due to secreted class III peroxidases produced by the plant, while *F. oxysporum* utilized components of a MAPK signaling pathway to respond to these environmental cues (278, 279). Key signaling components included *F. oxysporum* Ste2, a functional homolog of the *S. cerevisiae* α pheromone-sensing receptor. No sexual cycle has been observed in *F. oxysporum*, but the addition of synthetic α pheromone analogs (either *S. cerevisiae* or *F. oxysporum* pheromones) induced polarized growth in *F. oxysporum* cells. Loss of Ste2 blocked both the response to α pheromone analogs and the ability to respond to root peroxidases and also reduced the virulence on tomato plants. These results demonstrate a role for Ste2 and MAPK signaling in responding to environmental cues but generate additional questions, including how the same pheromone receptor can respond both to pheromone peptides and to plant peroxidases. This is particularly true given that peroxidases have to be catalytically active to elicit a chemotropic response in *F. oxysporum* (279). Further experiments will be needed to address these questions, but it appears clear that the pheromone-signaling machinery has been adapted for novel signaling purposes in select species.

Adaptation of the pheromone-sensing pathway is also represented by pheromone-induced biofilm formation in *C. albicans*. As discussed earlier, pheromone signaling not only induces polarized growth in responding cells, but also increases adherence of cells to inert surfaces and leads to the establishment of biofilms. These biofilms may have evolved to enhance mating of *C. albicans* cells in the mammalian host, or they could act to seed infections independent of their role in promoting the parasexual cycle.

It is also true that the transcriptional targets of *MAT* genes can include genes that are involved with processes other than sexual reproduction. In *C. albicans*, the white-opaque switch is directly regulated by the mating-type locus, but this switch regulates pleiotropic attributes in addition to sexual mating and biofilm formation. Similarly, in *P. chrysogenum*, the targets of MAT1-1-1 include genes with functions in asexual development; morphogenesis; and amino acid, iron, and secondary metabolism (277).

CONCLUSIONS

Given the breadth of *Ascomycetes*, the diversity of mating programs present in this phylum is perhaps not surprising. From the relatively simple programs in the model yeasts to the more complex programs in filamentous species, studies continue to highlight the variety of techniques used to support sexual reproduction. Furthermore, given recent exciting examples, it seems likely that other novel functions for pheromone-signaling pathways will be uncovered in fungal species and that these will similarly take advantage of the existing cellular machinery to enable adaptation during evolution.

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Fungal Sex: The Basidiomycota

7

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BREEDING SYSTEMS AND LIFESTYLES IN THE BASIDIOMYCOTA

In the phylum Basidiomycota, a wide variety of lifestyles are represented. These range from well-known and conspicuous wood-decaying mushrooms, plant growth-promoting and mutualistic mycorrhizae, and crop-destroying smut and rust fungi, to yeast-like human pathogens. Lifestyle differences have consequences for the mating and breeding systems of these fungi (see "Glossary," below, for definitions of specialist terms used in this article), which are reflected in the genetic evolution of mating-type determination. For over a century fungi have been recognized as having diverse breeding systems, from homothallism (i.e., universal compatibility among gametes, including among clonemates) to heterothallism (i.e., mating among haploid gametes carrying different mating-type alleles). The study of breeding systems, for example, led to the discovery of the astounding variability in mating-type alleles among mushrooms, with thousands of different mating types in some species (1), and to the realization that in many fungal pathogens the process of sexual reproduction is closely linked to infection and pathogenicity (2) (Fig. 1). The importance of basidiomycete fungi and their great research tractability, from ecology to genomics, have brought major insights into the diversification of genetic mechanisms used to achieve sexual reproduction.

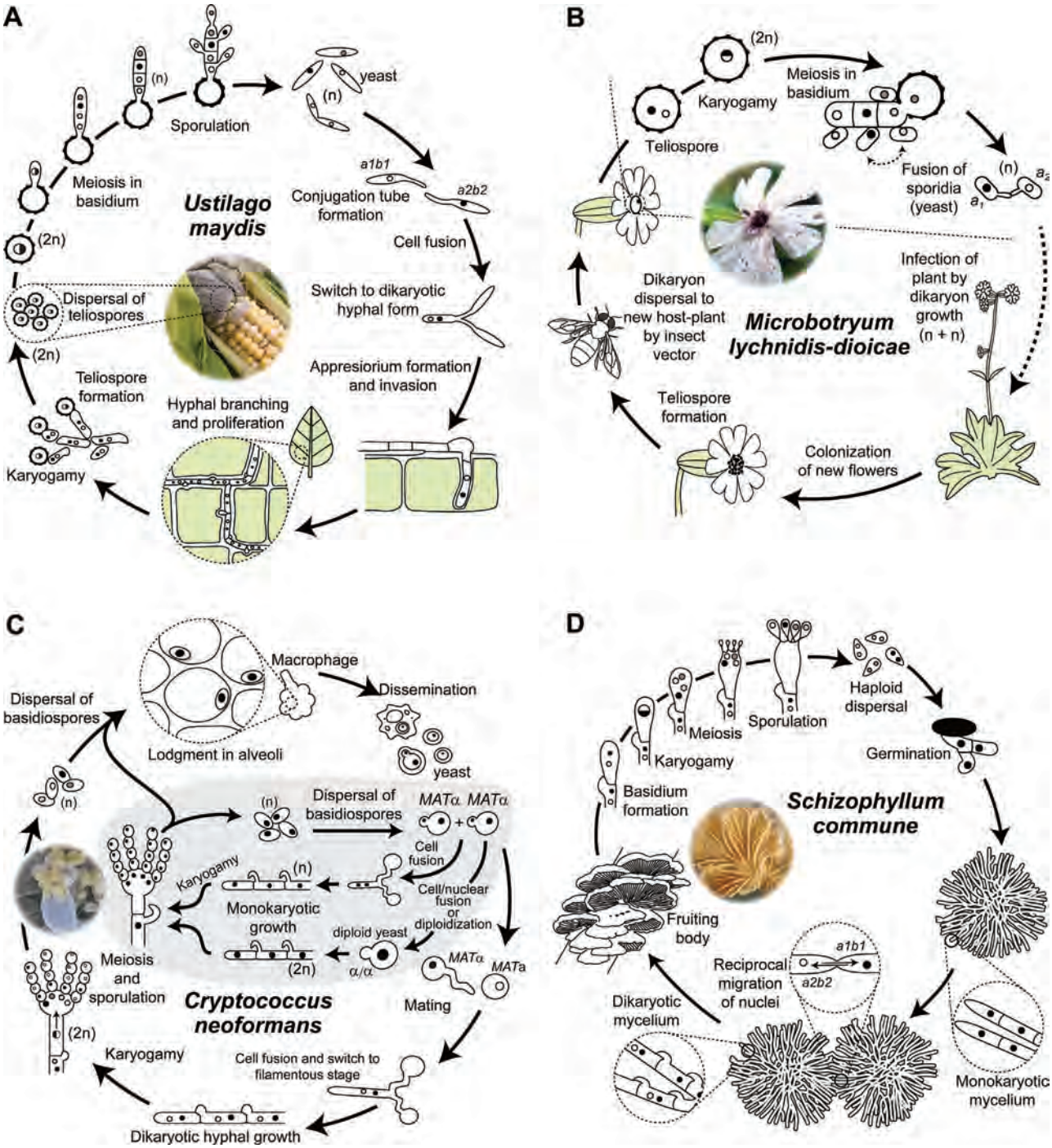
Diversity and Phylogenetic Relationships: Phylum Basidiomycota

From a phylogenetic perspective, the phylum Basidiomycota is the sister group to the phylum Ascomycota, forming together the subkingdom Dikarya. Three independently evolving lineages are strongly within the Basidiomycota (Fig. 2) (3). The subphylum Agaricomycotina contains most of the described species (ca. 21,000), including many mushrooms as saprophytes or mycorrhizal symbionts of plants, the jelly fungi, and a large diversity of yeasts, some of which are important pathogens of humans (*viz.*, *Cryptococcus neoformans*) (4, 5). The subphylum Ustilaginomycotina comprises more than 1,700 species, and while many species are pathogens of graminaceous plants (such as the maize smut *Ustilago maydis*), others are commonly associated with human and animal infections and are known from their asexual (anamorphic) states only (*viz.*, *Malassezia* spp.) (4, 6, 7). The subphylum Pucciniomycotina is a sister group of the clade containing Ustilaginomycotina and Agaricomycotina and consists of more than 8,400 described species (8). Besides the array of saprobic yeast species usually recovered from soils, aquatic habitats, or the phylloplane (e.g., *Rhodotorula* spp. and *Sporobolomyces* spp.) (9), most of the Pucciniomycotina species are plant parasites, such as the obligate pathogenic rust fungi (e.g., *Puccinia* spp.) or anther-smut fungi (*Microbotryum* spp.). Through the diversification of mating and dispersal stages, this huge variety of fungal lifestyles is highly integrated with equally diverse sexual cycles and breeding systems, a theme that has long been the subject of study by influential mycologists (1, 10–13).

Sexual Development and Determination of Cell Type Identity

Most fungi are able to undergo both asexual and sexual reproduction and have evolved tightly controlled mechanisms to regulate the process of mating, with respect

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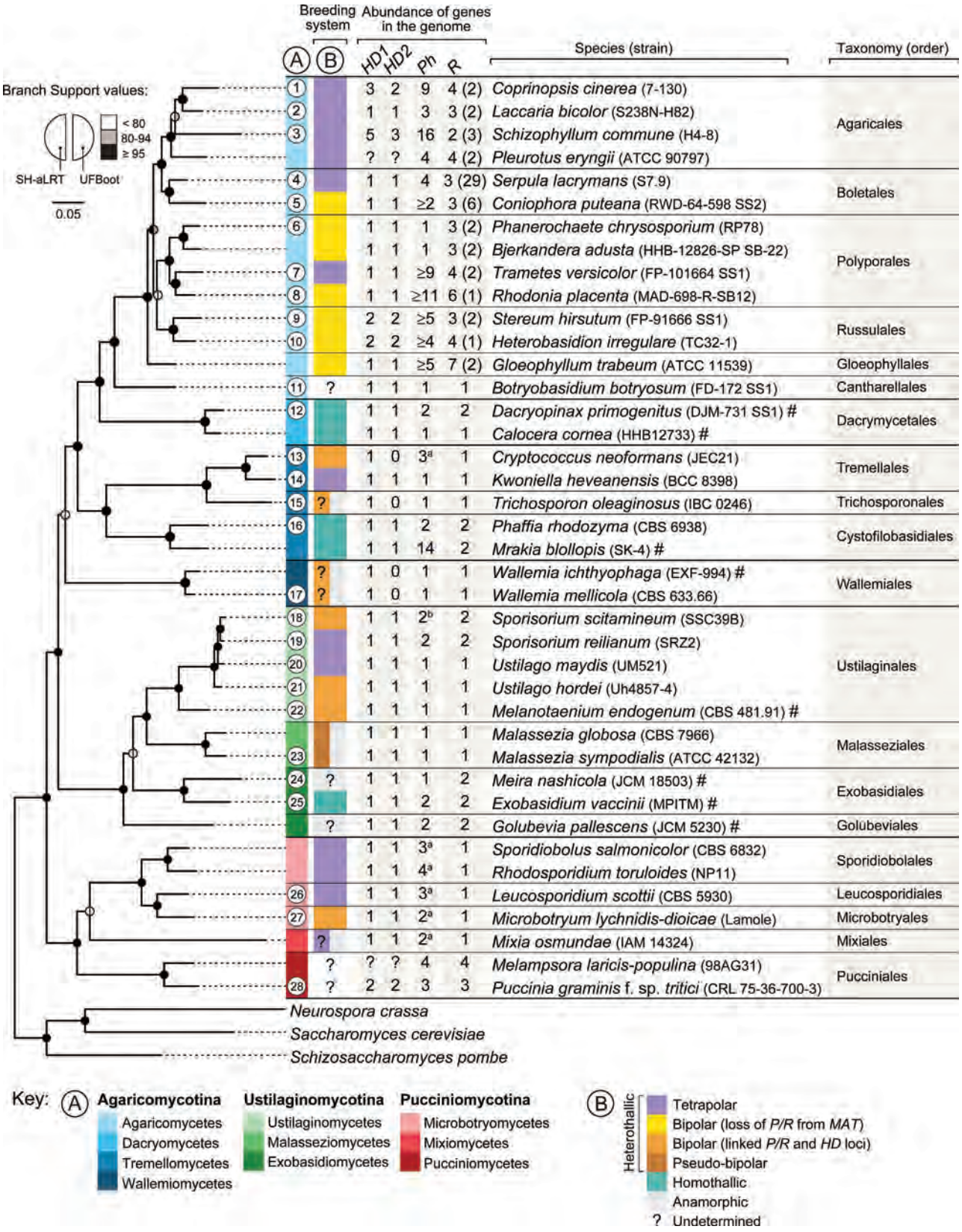

A
Ustilago maydis
(n)
Sporulation
yeast
(n)
a1b1
a2b2
Conjugation tube formation
Cell fusion
Switch to dikaryotic hyphal form
Appresiorium formation and invasion
Hyphal branching and proliferation
Meiosis in basidium
(2n)
Dispersal of teliospores
(2n)
Teliospore formation
Karyogamy
B
Microbotryum lychnidis-dioicae
(2n)
Karyogamy
Meiosis in basidium
Teliospore
Fusion of sporidia (yeast)
(n)
Infection of plant by dikaryon growth (n + n)
Dikaryon dispersal to new host-plant by insect vector
Teliospore formation
Colonization of new flowers
C
Cryptococcus neoformans
Macrophage
Dissemination
yeast
Lodgment in alveoli
Dispersal of basidiospores
(n)
(n)
Dispersal of basidiospores
MATα MATα
Cell fusion
Karyogamy
(n)
Monokaryotic growth
Cell/nuclear fusion or diploidization
diploid yeast
α/α
(2n)
MATα
MATa
Mating
Meiosis and sporulation
(2n)
Karyogamy
Cell fusion and switch to filamentous stage
Dikaryotic hyphal growth
D
Schizophyllum commune
Haploid dispersal
Sporulation
Meiosis
Karyogamy
Basidium formation
Germination
Fruiting body
a1b1
a2b2
Reciprocal migration of nuclei
Dikaryotic mycelium
Monokaryotic mycelium

to timing, and gamete dispersal, recognition, and fusion. In basidiomycetes, the sexual cycle typically involves fusion of genetically distinct homokaryotic hyphae or haploid yeast cells to produce a dikaryon, in which the two haploid parental nuclei are replicated in a coordinated fashion without fusion during hyphal elongation, usually involving the formation of clamp connections (i.e., a hook-like structure formed by hyphal cells to ensure proper distribution of the two genetically distinct nuclei during mitotic cell divisions; see below) (14). Nuclear fusion (karyogamy) then takes place in the basidia or in other specialized structures (e.g., teliospores), after which the diploid nucleus undergoes meiosis to generate haploid basidiospores (meiospores) and complete the life cycle (see Fig. 1 for representative life cycles).

Despite the wide variation of sexual cycles in nature, one common underlying feature shared by most fungi is the lack of genetically determined anisogamy: many fungi are isogamous (i.e., where all gametes have the same sizes), and even in anisogamous species, all haploid genotypes produce both types of gamete sizes. This means that there are not individuals of different sexes in fungi (15). Furthermore, many fungi are heterothallic, meaning that syngamy can only occur between gametes of different genetically determined mating types (15–17).

In members of the sister phylum Ascomycota, mating-type identity is governed at a single genetic mating type (*MAT*) locus (see reference 197). In that case, only two mating types segregate in meiosis, defining what is termed a bipolar system. By contrast, basidiomycetes have evolved a breeding system that relies on two genetic *MAT* loci. One locus encodes tightly linked pheromones and pheromone receptors (hereafter referred to as the *P/R* locus), and the other encodes homeodomain-type transcription factors (hereafter, *HD* locus), determining viability following syngamy. For successful mating and completion of the sexual cycle, haploid cells that conjugate must differ at both *MAT* loci (18, 19). When the two *MAT* loci are unlinked, four mating types can be generated by meiosis among the haploid progeny, defining this as a tetrapolar breeding system. Other basidiomycetes have instead a bipolar system controlled by a single *MAT* locus, either because the *P/R* and *HD* loci are linked or because one has lost its function in mating-type determinism. In mushroom-forming species (Agaricomycetes), there has been a generalized diversification of alleles at both *MAT* loci, in some cases yielding species with hundreds or thousands of possible mating types (1, 20–22). Data compiled from early studies indicate that as many as 65% of the species in the Agaricomycotina are tetrapolar (13, 23), whereas classical mating studies indicate a predominance of bipolar systems in the majority of the Ustilaginomycotina (24) and the Pucciniomycotina (25). In the following sections we summarize current knowledge of the

Figure 1 General life cycles of dimorphic and mushroom-forming basidiomycetes. Three basidiomycetes are pictured where sexual reproduction and a dimorphic switch between a yeast cell and a hyphal form are crucial to infection of plant (A, B) or animal (C) hosts. The haploid yeast forms of the maize smut *Ustilago maydis* (A) and the anther smut *Microbotryum* spp. (B) are nonpathogenic and can undergo asexual mitotic vegetative growth. In *Microbotryum*, the yeast stage is, however, short-lived because mating occurs mostly between cells within the same tetrad. Upon mating with a compatible partner, both fungi switch to an enduring infection hyphal form (dikaryon; n + n) that can invade the host plant. Proliferation and differentiation of *U. maydis* (**A**) in the plant culminates with the production of masses of wind-dispersing diploid spores (teliospores; 2n) in large tumor-like tissues, whereas in *Microbotryum* (**B**), teliospores are formed in the anthers of infected flowers and transmitted by pollinators onto healthy plants. In the case of *Cryptococcus neoformans* (**C**), the single-celled yeast form may be free-living or mycoparasitic. A similar dimorphic switch occurs upon mating of yeast cells of opposite mating type (**α** or α), ultimately resulting in the infectious propagules (basidiospores) that potentially infect an animal host after dispersal. These infectious structures may also be generated by haploid selfing (depicted with gray background), where fusion occurs between homothallic cells carrying identical *MAT* alleles (α/α diploid is depicted) and form monokaryotic hyphae with unfused clamp connections (see text for details). In mushroom-forming fungi such as *Schizophyllum commune* (**D**), germination of haploid spores yields haploid monokaryons capable of independent growth. When two compatible monokaryons meet, a fertile clamped dikaryon is formed which develops into fruiting bodies (mushrooms) triggered upon suitable environmental cues, where basidia arise. In all these and other basidiomycetes, nuclear fusion (karyogamy) is usually delayed until the formation of basidia (or teliospores). Meiosis ensues, generating four haploid nuclei, which give rise to basidiospores to complete the cycle. Adapted from Morrow and Fraser (2) and Nieuwenhuis et al. (17) with permission of the publishers.


Breeding system
Abundance of genes in the genome
A
B
HD1
HD2
Ph
R
Species (strain)
Taxonomy (order)
Branch Support values:
< 80
80-94
≥ 95
SH-aLRT
UFBoot
0.05
1 3 2 9 4 (2) Coprinopsis cinerea (7-130)
2 1 1 3 3 (2) Laccaria bicolor (S238N-H82)
3 5 3 16 2 (3) Schizophyllum commune (H4-8)
? ? 4 4 (2) Pleurotus eryngii (ATCC 90797)
Agaricales
4 1 1 4 3 (29) Serpula lacrymans (S7.9)
5 1 1 ≥2 3 (6) Coniophora puteana (RWD-64-598 SS2)
Boletales
6 1 1 1 3 (2) Phanerochaete chrysosporium (RP78)
1 1 1 3 (2) Bjerkandera adusta (HHB-12826-SP SB-22)
7 1 1 ≥9 4 (2) Trametes versicolor (FP-101664 SS1)
8 1 1 ≥11 6 (1) Rhodonia placenta (MAD-698-R-SB12)
Polyporales
9 2 2 ≥5 3 (2) Stereum hirsutum (FP-91666 SS1)
10 2 2 ≥4 4 (1) Heterobasidion irregulare (TC32-1)
Russulales
1 1 ≥5 7 (2) Gloeophyllum trabeum (ATCC 11539)
Gloeophyllales
11 ? 1 1 1 1 Botryobasidium botryosum (FD-172 SS1)
Cantharellales
12 1 1 2 2 Dacryopinax primogenitus (DJM-731 SS1) #
1 1 1 1 Calocera cornea (HHB12733) #
Dacrymycetales
13 1 0 3a 1 Cryptococcus neoformans (JEC21)
14 1 1 1 1 Kwoniella heveanensis (BCC 8398)
Tremellales
15 ? 1 0 1 1 Trichosporon oleaginosus (IBC 0246)
Trichosporonales
16 1 1 2 2 Phaffia rhodozyma (CBS 6938)
1 1 14 2 Mrakia blollopis (SK-4) #
Cystofilobasidiales
? 1 0 1 1 Wallemia ichthyophaga (EXF-994) #
17 ? 1 0 1 1 Wallemia mellicola (CBS 633.66)
Wallemiales
18 1 1 2b 2 Sporisorium scitamineum (SSC39B)
19 1 1 2 2 Sporisorium reilianum (SRZ2)
20 1 1 1 1 Ustilago maydis (UM521)
21 1 1 1 1 Ustilago hordei (Uh4857-4)
22 1 1 1 1 Melanotaenium endogenum (CBS 481.91) #
Ustilaginales
1 1 1 1 Malassezia globosa (CBS 7966)
23 1 1 1 1 Malassezia sympodialis (ATCC 42132)
Malasseziales
24 ? 1 1 1 2 Meira nashicola (JCM 18503) #
25 1 1 2 2 Exobasidium vaccinii (MPITM) #
Exobasidiales
? 1 1 2 2 Golubevia pallescens (JCM 5230) #
Golubeviales
1 1 3a 1 Sporidiobolus salmonicolor (CBS 6832)
1 1 4a 1 Rhodosporidium toruloides (NP11)
Sporidiobolales
26 1 1 3a 1 Leucosporidium scottii (CBS 5930)
Leucosporidiales
27 1 1 2a 1 Microbotryum lychnidis-dioicae (Lamole)
Microbotryales
? 1 1 2a 1 Mixia osmundae (IAM 14324)
Mixiales
? ? ? 4 4 Melampsora laricis-populina (98AG31)
28 ? 2 2 3 3 Puccinia graminis f. sp. tritici (CRL 75-36-700-3)
Pucciniales
Neurospora crassa
Saccharomyces cerevisiae
Schizosaccharomyces pombe
Key:
A
Agaricomycotina
Agaricomycetes
Dacryomycetes
Tremellomycetes
Wallemiomycetes
Ustilaginomycotina
Ustilaginomycetes
Malasseziomycetes
Exobasidiomycetes
Pucciniomycotina
Microbotryomycetes
Mixiomycetes
Pucciniomycetes
B
Heterothallic
Tetrapolar
Bipolar (loss of P/R from MAT)
Bipolar (linked P/R and HD loci)
Pseudo-bipolar
Homothallic
Anamorphic
? Undetermined

molecular basis of mating-type determination in basidiomycetes and discuss transitions in breeding systems, using examples from throughout the Basidiomycota phylogenetic diversity.

MOLECULAR DETERMINANTS OF MATING TYPE

The Pheromone/Receptor Sensing System

In the Basidiomycota, syngamy is governed by, in its simplest form, small 10- to 15-amino acid-lipopeptide pheromones derived from 35 to 40 amino acid precursors through posttranslation modifications at both the N- and C-termini (26–28). These diffusible pheromones are received by seven transmembrane-domain pheromone receptors, coupled to a G-protein for downstream signal transduction (28, 29). This molecular determination of mating fusion is similar in part to the **a**- and α-factor P/R system in the ascomycetes and is thought to predate the separation of Asco- and Basidiomycota lineages. There is, however, one remarkable difference between the phyla: only homologues of genes encoding the pair of the **a**-factor pheromone and Ste3 pheromone receptor of the ascomycete system appear to exist in basidiomycetes. Therefore, instead of the **a**-factor/Ste3 and α-factor/Ste2 coupled sensing system characteristic of the ascomycetes (described in reference 197), "chemo-sensing" specificity in basidiomycetes is mediated by allelic variants of the same type of genes (18). Mating is often initiated by a reciprocal exchange of pheromones recognized by matching pheromone receptor variants in both mating types, and thus two strains need to carry different alleles of the pheromone and receptor genes at the *P/R* locus.

All active basidiomycete mating pheromones isolated so far are hydrophobic diffusible lipopeptides that undergo farnesylation at the C-terminal cysteine residue of the CAAX motif (where "C" is cysteine, "A" is often an aliphatic amino acid, and "X" is any residue) and amino-terminal processing. This was first demonstrated in the secreted pheromone (rhodotorucine A) of the red-pigmented yeast *Rhodosporidium toruloides* (recently renamed *Rhodotorula toruloides*) (30, 31) and later extended to many other basidiomycetes (26, 32–36), and also for the **a**-factor of the ascomycete yeast *Saccharomyces cerevisiae* that now stands as the model paradigm for the fungal kingdom (37, 38).

Among the basidiomycetes, exceptional pheromone gene structures were revealed in members of the Pucciniomycotina. Where investigated, the pheromone precursor genes each encode tandem copies of the mature peptide moiety (39–43), and an initial step of processing of a spacer region is required to reveal C-terminal CAAX motifs for each repeat (37, 44). In species where pheromones of opposite mating types have been clearly identified (namely, in *R. toruloides*, *Sporidiobolus salmonicolor*, *Leucosporidium scottii*, and *Microbotryum* spp.), the *MAT A1* pheromone precursor typically presents a greater number of repeats of the peptide moiety (up to six copies in some species), whereas the *MAT A2* pheromone gene appears to encode only one or two copies of the mature pheromone (39, 42, 43, 45). Interestingly, this increased production of pheromone per mole of precursor in *MAT A1* cells, possibly allowing communication with *A2* cells at longer distances, is consistent with the observed asymmetric growth of conjugation tubes in both *R. toruloides* (46) and *Microbotryum* spp. (43, 47), which occurs earlier and to a greater extent from *MAT A2* cells. These

Figure 2 Phylogeny of the Basidiomycota indicating the breeding system and the number of *MAT* genes across representative species of the three subphyla. The breeding system and the different taxonomic lineages are color-coded as given in the key and are kept consistent in all figures. Gene numbers shown for each species were obtained either from previous reports (20, 41, 100) or from newly surveyed genome data (marked with a hash sign after the species name). In the Agaricomycetes, values shown in parentheses are putative nonmating-type pheromone receptors. A question mark indicates cases where information on the breeding system is not available or is uncertain (e.g., because the sexual stage of a species is unknown). A schematic representation of the *P/R* and *HD* loci is given in Fig. 3 for representative species of each lineage marked with numbers enclosed in white circles. Letters in superscript next to the number of pheromone precursor genes indicate that (a) all genes encode the same mature pheromone peptide or that (b) no CAAX motif was detected in one of the putative pheromone precursors. The species phylogenetic tree was constructed in IQ-TREE (193) using a previously described approach (194). Branch support values are shown in the tree nodes as given in the key and were assessed with the ultrafast bootstrap approximation (UFBoot) and the approximate likelihood ratio test (SH-aLRT), each with 1,000 replicates. The basidiomycete clade is rooted with sequences from Ascomycete fungi.

findings suggest that the two mating types may have distinct signaling roles during conjugation, despite the absence of morphologically differentiated cells.

In response to the interaction of the pheromone ligand with a suitable pheromone receptor, mating and sexual development are initiated through a heterotrimeric G protein located at the plasma membrane that triggers a variety of downstream signaling processes. In *U. maydis*, where this has been investigated in greater detail, this signaling is attributed to two interconnected pathways involving a cAMP-dependent protein kinase A and a mitogen-activated protein kinase (28, 48–50). The point of convergence of the two pathways is the pheromone response factor (Prf1), which is an HMG-box transcription factor that recognizes and binds to pheromone response elements located in the regulatory regions of pheromone-induced genes (51, 52). Interestingly, whereas most of the core components of the mitogen-activated protein kinase cascade pathway (e.g., Ste11, Ste7, Fus3) are conserved over wide evolutionary distances, the key downstream transcription factors that ultimately activate or repress their target genes are often not conserved among species and thus are difficult to reveal through candidate gene approaches or sequence comparison.

Homeodomain Transcription Factors: The Second Compatibility Checkpoint

While the *P/R* system controls syngamy, the production of viable mating products requires compatibility at additional genetic components in the basidiomycetes, which is mediated by genes at the *HD* locus. In its most basic configuration, the *HD* locus encodes a pair of proteins of dissimilar homeodomain classes, having protein domains HD1 or HD2, and their coding genes are normally adjacent and divergently transcribed. Development following syngamy depends upon the formation of functional heterodimeric transcription factors where dimerization is restricted to HD1 and HD2 proteins that originate from haploid mates bearing different alleles of the *HD* locus. This requirement ensures that active heterodimers are only formed in the dikaryotic stage and do not arise in haploid cells. Failure to form heterodimers in the haploid is explained by interfering amino acids at HD1 and HD2 proteins from the same allele that disturb their general cohesiveness (53–55). Studies have shown that the relevant interfaces for discriminating HD1-HD2 interactions and determining allele specificity seem to be located N-terminally to the DNA binding motifs and that this region is usually highly variable between different alleles of both *HD1* and *HD2* genes (53, 54, 56). Analyses in *U. maydis* of chimeric and mutant alleles and protein-protein interaction studies using the yeast two-hybrid system revealed that dimerization between HD1 and HD2 proteins involves polar-hydrophobic interactions with variable cohesive contact sites contributing to binding affinity (54, 57, 58). Once formed, the functional heterodimers will bind to gene promoter elements, leading to the induction of the specific sets of genes involved in subsequent differentiation, which can include a morphological switch from yeast-like cells to filamentous growth and pathogenicity, as in the smuts, or fruiting body formation in the mushrooms (28, 41, 58–60).

BREEDING SYSTEMS IN THE BASIDIOMYCOTA

The distinction between heterothallic and homothallic compatibility represents a fundamental breeding system classification in fungi, and both systems are found across the Basidiomycota. In heterothallic basidiomycete species, sexual reproduction is only possible between gametes carrying different alleles at both *MAT* loci, preventing intrahaploid mating (i.e., selfing at the haploid level) (61). On the other hand, homothallic species produce universally compatible gametes, each being able to undergo intrahaploid mating with their clonemates. We have summarized in Fig. 2 and Fig. 3 data regarding the breeding system and the genomic organization of the mating-type genes in representative species of the three major subphyla of the Basidiomycota, some of which will be highlighted in the following sections.

Tetrapolar Systems: *U. maydis* and Other Smut Relatives

The maize smut *U. maydis* (order Ustilaginales; Fig. 2) is one of the most intensively studied basidiomycete models with respect to the organization and function of mating-type genes (62). The fungus has a dimorphic life cycle with a yeast-like haploid saprophytic phase switching to filamentous pathogenic growth in the dikaryotic stage following syngamy (Fig. 1A). In this species, the process of mating and completion of the sexual cycle is regulated by a tetrapolar breeding system, i.e., with the *P/R* (or *a*) locus unlinked from the *HD* (or *b*) locus. The *P/R* locus exists in two allelic forms (*a1* and *a2*), which are defined by structurally dissimilar DNA regions of 4.5 kb for the *a1* and 8 kb for the *a2*, each comprising one pheromone receptor gene (*PRA1* or *PRA2*) and one pheromone precursor gene (*MFA1* or *MFA2*) (Fig. 3B). The Pra1 receptor only responds to the Mfa2 pheromone, while Pra2 can

only recognize Mfa1. The observed size difference between the two allelic forms of the *P/R* locus is due to the presence in the *a2* allele of two additional genes (*lga2* and *rga2*) that direct uniparental mitochondrial DNA inheritance, as well as a third, nonfunctional pheromone-encoding gene (26, 33, 52, 63).

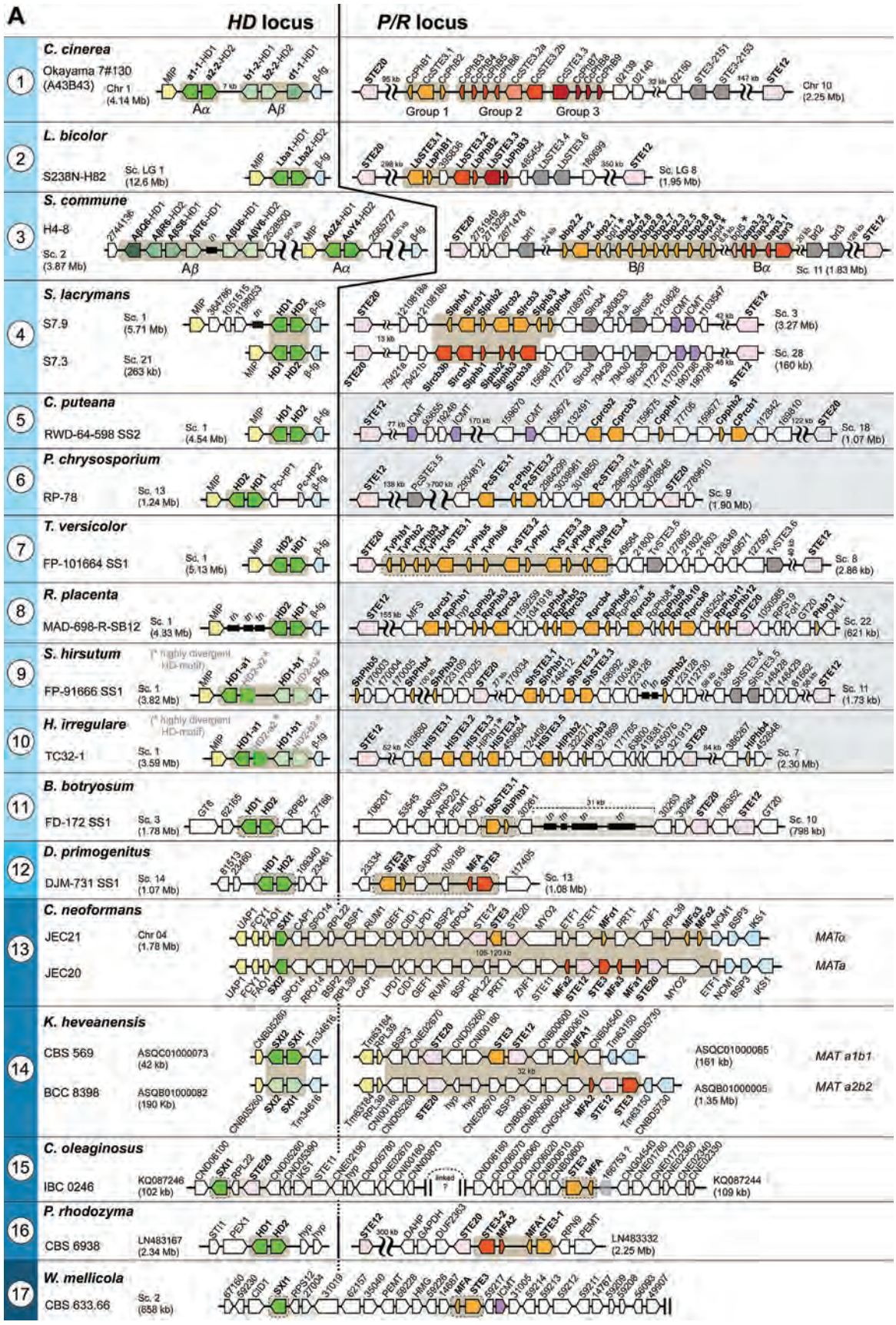
Interestingly, the presence of this pseudogene led to early speculations about the existence of a complex scenario ancestral to the Ustilaginales in which the *P/R* locus occurred in more than two allelic forms, with this additional cryptic pheromone gene being recognized by a distinct receptor encoded by a third unidentified *P/R* allele (52). This hypothesis was substantiated upon characterization of the breeding system of the closely related head smut fungus *Sporisorium reilianum*. Compared to *U. maydis*, the *P/R* locus of *S. reilianum* exists in three alleles, each containing two distinct pheromone genes and one pheromone receptor (64) (Fig. 3B). Importantly, each of the mature pheromones in one allele interacts specifically with only one of the receptors encoded by the other two alleles. For instance, the pheromones encoded by the *MFA1.2* and *MFA1.3* genes at the *a1* locus interact with the Pra2 and the Pra3 receptors encoded by the *a2* and *a3* alleles, respectively (Fig. 3B). This leads to a scenario in which each mating type can detect (and be detected by) the other two, but not itself. Additional support for this ancestral triallelic *P/R* system model was more recently attained in a study where the complete *P/R* loci of additional smut species spanning about 100 million years of evolution in the order Ustilaginales were sequenced and compared (65). Interestingly, the three *P/R* alleles previously described in *S. reilianum* were conserved and generally syntenic among members of the same order. This supports a potential scenario in which the biallelic *P/R* system as observed in *U. maydis* resulted from the loss of the *a3* allele and loss of one of the two pheromone genes, which is now lacking in extant *a1* and *a2* alleles. Accordingly, a phylogenetic analysis using pheromone receptor sequences from a wider taxonomic range (Fig. 4) shows that all Ustilaginomycotina receptors group together and within one of the three allelic classes previously defined (*PRA1*, *PRA2*, *PRA3*) (65). This result denotes that the triallelic *P/R* system is probably as old as the stem age of the Ustilaginomycotina, about 450 million years (66).

Following fusion of compatible partners, heterozygosity at the *HD* locus is required for dikaryon maintenance and filamentous growth in *U. maydis* (54, 67). The two divergently transcribed *HD* genes encode homeodomain proteins known as bE (for *bEast*; HD1-type) and bW (for *bWest*; HD2-type). However, in contrast to the biallelic and triallelic *P/R* locus of *U. maydis* and *S. reilianum*, respectively, the *HD* locus is highly multiallelic: in *U. maydis* more than 30 intercompatible allelic versions were found in nature (68; DeVay, cited in 69), while in *S. reilianum* five alleles have been identified so far (64). The homeodomains and C-terminal regions of the allelic HD1 and HD2 proteins are highly conserved, whereas the sequences N-terminal to the homeodomain regions are highly variable and required for the interaction of nonallelic HD1 and HD2 proteins (54).

Variation in Tetrapolar Systems beyond *Ustilago/Sporisorium*

Outside the Ustilaginomycotina, the number of tetrapolar species characterized for molecular polymorphisms at the *MAT* loci has increased in recent years (42, 45, 70–74). *Kwoniella heveanensis* (70) and *Cryptococcus amylolentus* (71) in the Agaricomycotina and *L. scottii* (42) in the Pucciniomycotina are examples where the *P/R* locus is biallelic, while the *HD* locus displays greater polymorphism (Fig. 2 and 3). In all three, the *P/R* and *HD* loci localize to different chromosomes. Sequencing of the *P/R* locus in each species showed their biallelic nature but, in comparison to *U. maydis* and *S. reilianum*, the *P/R* locus in *K. heveanensis* and *L. scottii* covers a relatively larger genomic region (about 32 kb and 50 kb, respectively; Fig. 3A and 3B), which is highly rearranged between mating types. Included here are the genes encoding *MAT*-specific pheromones (*MFA* or *RHA*) and receptors (*STE3*), as well as genes previously shown to be required for the onset of filamentation in dimorphic species (e.g., *STE20*, *STE12*) (75–77) and lineage-specific genes not obviously involved in mating (Fig. 3). The *HD* locus in *K. heveanensis* and *C. amylolentus* contains only the divergently transcribed homeodomain genes, *HD1* and *HD2* (named *SXI1* and *SXI2*) (70, 71). In *L. scottii*, in contrast to *K. heveanensis* and *C. amylolentus*, the boundaries of the *HD* locus may have extended further outside from the *HD1/HD2* gene module, given the evidence of increased sequence divergence in a region spanning about ~80 kb that encompasses the *HD1/HD2* genes (42). Sequence analysis of this variable region from different *K. heveanensis* isolates provided evidence that the locus is multiallelic with at least six different *HD1/HD2* alleles (70). The same holds true for *L. scottii*, but with 28 *HD* alleles being revealed upon inspection of 43 isolates (42).

From an evolutionary standpoint, it is noteworthy that extant tetrapolar dimorphic basidiomycetes have more commonly a biallelic *P/R* locus (26, 70–72, 74,


A
HD locus
P/R locus
1
C. cinerea
Okayama 7#130
(A43B43)
Chr 1
(4.14 Mb)
Aα
Aβ
Group 1
Group 2
Group 3
Chr 10
(2.25 Mb)
2
L. bicolor
S238N-H82
Sc. LG 1
(12.6 Mb)
Sc. LG 8
(1.95 Mb)
3
S. commune
H4-8
Sc. 2
(3.87 Mb)
Aβ
Aα
Bβ
Bα
Sc. 11 (1.83 Mb)
4
S. lacrymans
S7.9
Sc. 1
(5.71 Mb)
S7.3
Sc. 21
(263 kb)
Sc. 3
(3.27 Mb)
Sc. 28
(160 kb)
5
C. puteana
RWD-64-598 SS2
Sc. 1
(4.54 Mb)
Sc. 18
(1.07 Mb)
6
P. chrysosporium
RP-78
Sc. 13
(1.24 Mb)
Sc. 9
(1.90 Mb)
7
T. versicolor
FP-101664 SS1
Sc. 1
(5.13 Mb)
Sc. 8
(2.86 kb)
8
R. placenta
MAD-698-R-SB12
Sc. 1
(4.33 Mb)
Sc. 22
(621 kb)
9
S. hirsutum
(* highly divergent HD-motif)
FP-91666 SS1
Sc. 1
(3.82 Mb)
Sc. 11
(1.73 kb)
10
H. irregulare
(* highly divergent HD-motif)
TC32-1
Sc. 1
(3.59 Mb)
Sc. 7
(2.30 Mb)
11
B. botryosum
FD-172 SS1
Sc. 3
(1.78 Mb)
Sc. 10
(798 kb)
12
D. primogenitus
DJM-731 SS1
Sc. 14
(1.07 Mb)
Sc. 13
(1.08 Mb)
13
C. neoformans
JEC21
Chr 04
(1.78 Mb)
JEC20
105-120 kb
MATα
MATa
14
K. heveanensis
CBS 569
ASQC01000073
(42 kb)
BCC 8398
ASQB01000082
(190 Kb)
32 kb
ASQC01000065
(161 kb)
ASQB01000005
(1.35 Mb)
MAT a1b1
MAT a2b2
15
C. oleaginosus
IBC 0246
KQ087246
(102 kb)
linked ?
KQ087244
(109 kb)
16
P. rhodozyma
CBS 6938
LN483167
(2.34 Mb)
LN483332
(2.25 Mb)
17
W. mellicola
CBS 633.66
Sc. 2
(658 kb)

78). For these, the proportion of compatible mates in the population cannot exceed 50%, irrespective of the number of alleles at the *HD* locus. However, given that compatibility at the *HD* locus can only be assessed after syngamy, increased *HD* allele diversity should be naturally selected when outcrossing is the rule, to allow more frequent dikaryon viability after syngamy and, in this way, limit the amount of wasted mating opportunities.

Tetrapolar Systems with Multiple Alleles in the Agaricomycetes

A vast repertoire of mating types has been found in most members of the class Agaricomycetes (Fig. 2), such as the wood-rotting mushroom *Schizophyllum commune* (Fig. 1D) with more than 15,000 intercompatible mating types, and the ink cap mushroom *Coprinopsis cinerea* with an estimated 12,000 distinct mating types (1). Such diversity is likely due to selection for rare alleles under outcrossing: any new specificity will be compatible with all extant mating types and therefore may increase in frequency under this selective advantage (15, 79). Studies have shown that two hallmarks of the *MAT* loci of these and other heterothallic Agaricomycetes account for such astonishing numbers of mating types within a single species. First, a single locus may itself be extremely polymorphic, in some cases with hundreds of specificities (1, 13, 16); second, for some species, multiallelism in both *P/R* and *HD* loci is generated by rounds of segmental duplication and diversification of *MAT* genes, resulting in independent but functionally redundant subloci, upon which recombination can act to give rise to novel allele specificities and, by definition, new mating types (80).

Recent advances in genome sequencing are now allowing detailed comparisons of *MAT* loci among diverse species, and a comparative overview of these genomic regions in several Agaricomycetes is presented in Fig. 3A. A great body of literature exists on the mating-type systems and distribution in Agaricomycetes, preventing a full discussion, and the reader is referred to some exceptional and recent reviews (16, 20). Here, we shall concentrate mainly on the model mushroom *C. cinerea*, which has long served as an invaluable model to understand the molecular underpinnings of multiple mating types and multiallelic *MAT* loci in the Agaricomycetes. Where relevant, reference will be made to other mushroom species, but many of the principles that govern the evolution of multiple mating types in *C. cinerea* seem also to hold in other species.

Initial studies in *C. cinerea* using classical genetics determined that the *HD* locus is multiallelic and composed of two subloci (*A* and *A*; Fig. 3A) that can recombine at detectable frequencies despite their close proximity in the genome (81, 82). Molecular genetic studies and genome sequencing later revealed that, whereas the *A* sublocus encodes one *HD1/HD2* gene pair, the *A* sublocus encodes up to three pairs and is located at a distance of ~7 kb from the *Aα* sublocus (Fig. 3A) (83–86). Recombination between the two subloci is possible through the ~7 kb-long intergenic

Figure 3 Schematic showing the genomic structure and diversity of *MAT* loci in representative basidiomycete lineages. The genomic organization of the homeodomain (*HD*) and pheromone/receptor (*P/R*) *MAT* loci is shown for selected species of (**A**) the Agaricomycotina and (**B**) the Ustilaginomycotina and Pucciniomycotina. Arrows indicate genes and their direction of transcription. Putative *MAT* loci are shaded in light brown, and *MAT* genes are colored as indicated in the key with different color grades representing different alleles (or paralogs). When known, conserved genes flanking *MAT* loci (colored light yellow or light blue) are shown within each lineage. Genes that encode components of the pheromone response pathway are shown in pink and are in many cases within the *MAT* locus. Putative homologs of a protein required for posttranslational modification of pheromone precursors (isoprenyl cysteine methyltransferase [ICMT]) are colored purple and appear near the *P/R* locus in some species. *P/R* loci no longer determining mating-type specificity in bipolar Agaricomycetes are depicted with a gray background. In *M. lychnidis-dioicae*, the two mating-type chromosomes are highly rearranged and enriched in transposable elements, so that only a small number of genes is depicted. Of note, whereas in *M. nashicola P/R* and *HD* genes are far apart on the same chromosome, in *E. vaccinii* and *M. endogenum* the two sets of genes are closer together. Other genes or genomic features are colored or represented as given in the key. For citations and additional details, see text. Gene names or their associated protein accession numbers are shown as they appear in their respective genome databases, except in species of Ustilaginomycotina and Tremellomycetes, where names were given based on sequence identity to the closest homolog in *U. maydis* and *C. neoformans*, respectively.

Figure 3 *continues on next page*

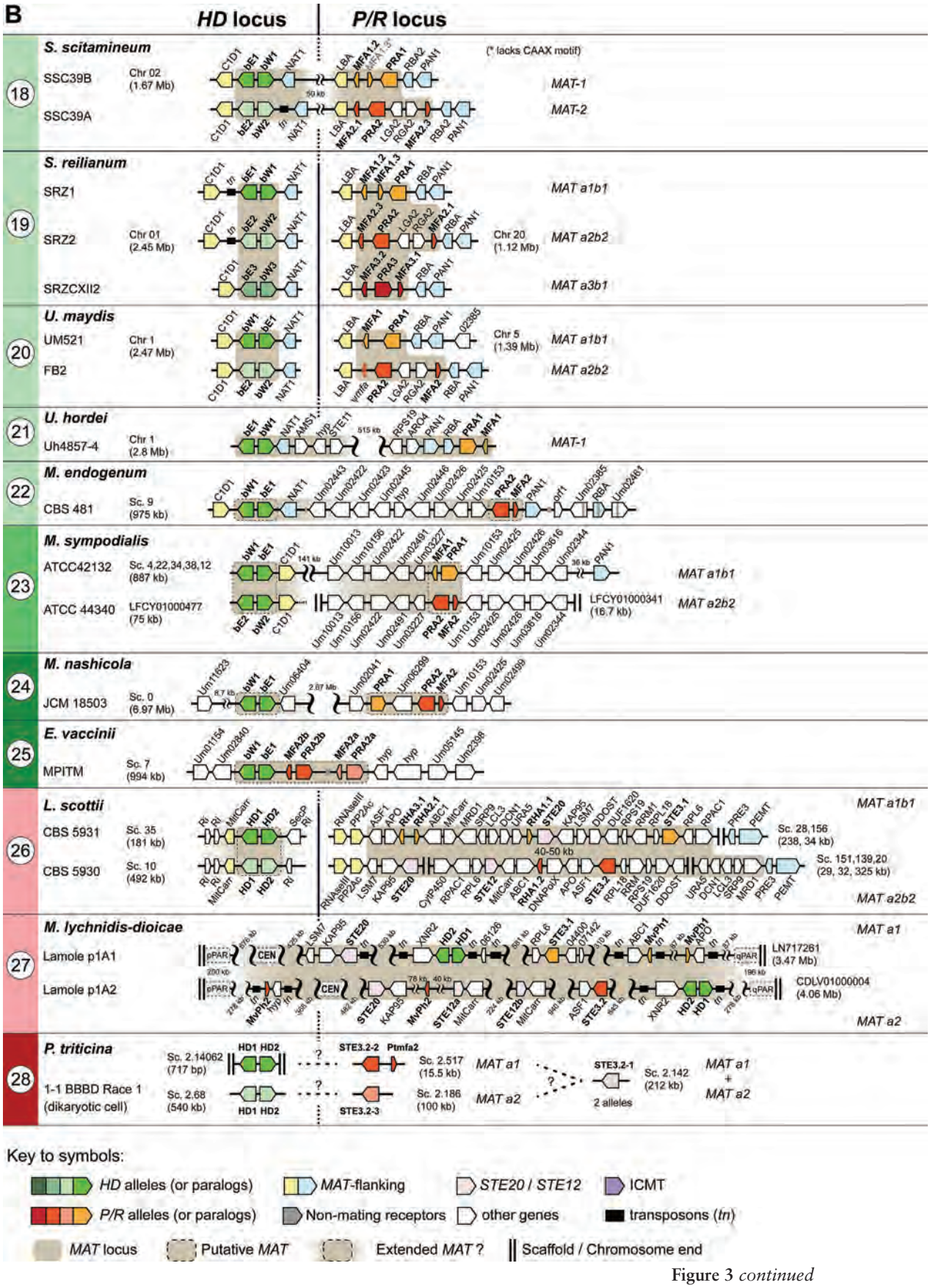


Figure 3 *continued*

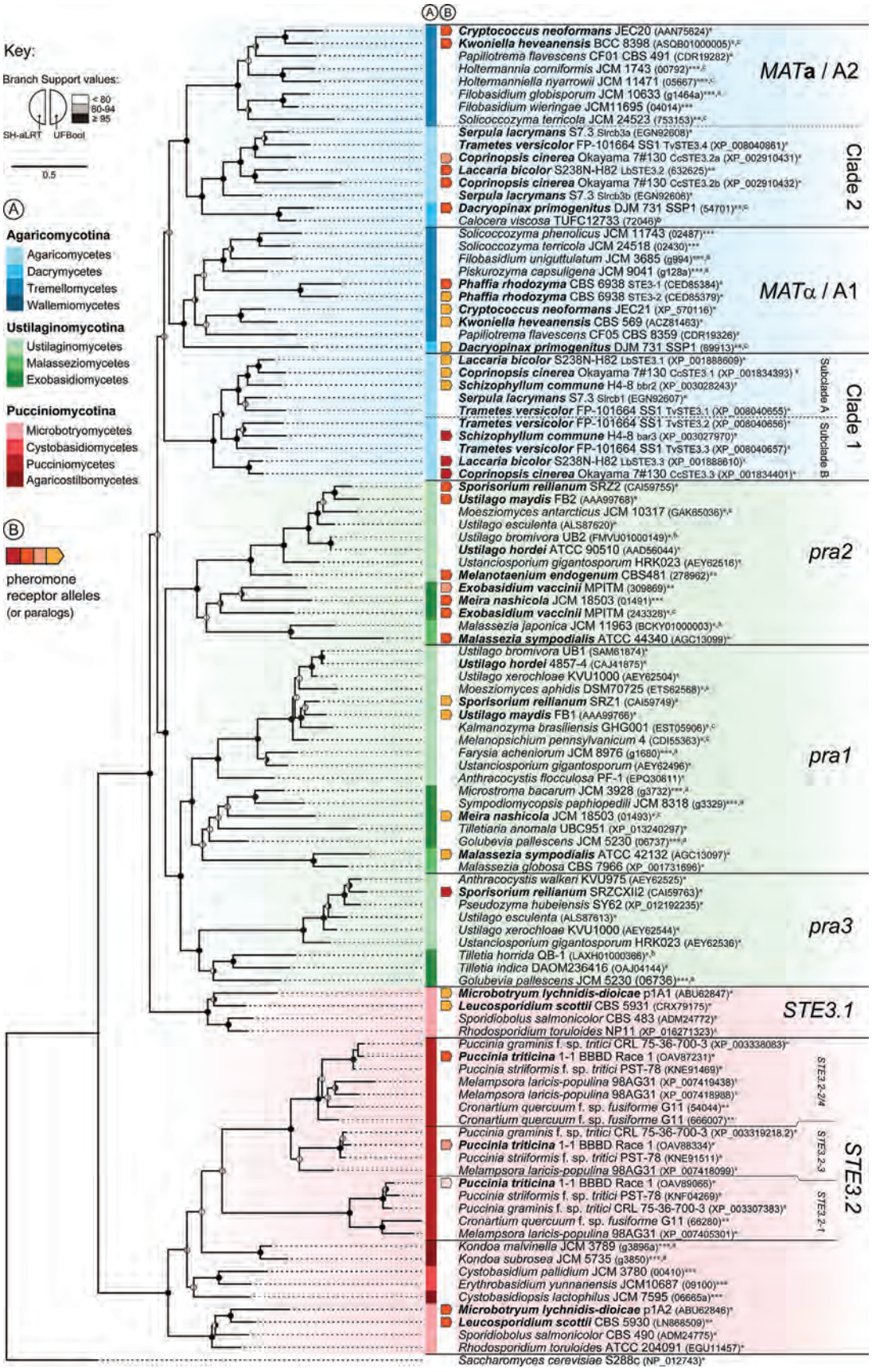
region that is highly conserved between alleles. Sequence and functional analysis of five *HD* alleles led to a theoretical archetypal structure for the *C. cinerea HD* locus with three paralogous *HD1/HD2* gene pairs (known as the *a*, *b*, and *d* pairs). This implies that molecular mechanisms akin to gene deletion have been at work during evolution of the *HD* locus, because the extant alleles have fewer than the six *HD1/HD2* genes postulated in the model (exemplified by allele *A43* in Fig. 3A) (83–85). At present, four, seven, and three distinct alleles have been identified for the *a, b*, and *d* pairs, respectively (56, 85), generating 84 *HD* alleles considering all possible permutations. Although this is about half the number estimated by John Raper, 160 *HD* alleles in nature (1), the identification of one additional allele per group would be sufficient to reach Raper's estimates. However, the actual numbers of alleles in nature are of course unknown.

Similarly, the *P/R* locus in *C. cinerea* is multiallelic and consists of three tandemly arranged but independent groups of paralogous genes (groups or subloci 1 to 3). The sequencing of several alleles revealed that each group contains multiallelic and functionally redundant genes encoding at least one pheromone receptor and up to four pheromone genes (Fig. 3A) (20). In any given group of paralogous genes, the relative order, direction of transcription, and number of genes may vary and, importantly, each group has freely interchangeable alleles (80). In *C. cinerea*, from 13 sequenced *P/R* alleles, two, five, and seven alleles were identified for groups 1, 2, and 3, respectively. Together, this gives rise to 70 possible combinations, approaching the number of 79 *P/R* alleles estimated to exist in nature (1). Phylogenetic analyses of *C. cinerea* receptors of the three paralogous gene groups and from different alleles added yet another layer of complexity in the evolutionary history. Strikingly, the different allelic versions of the pheromone receptors as defined by their position within the *P/R* locus do not always fall into the same clade (20, 80), an observation that led some authors to conclude that gene shuffling occurred within the locus during evolution (80). This pattern has also been observed in *S. commune* (87) and in *Ganoderma lucidum* (88). Such relocations may have restricted intralocus recombination at some point during evolution, thereby contributing to the fixation of some of the alleles in the populations.

With additional pheromone receptors from other Agaricomycetes added to the phylogenetic analysis, the origin of the paralogous groups of genes within the *P/R* locus can be traced back to an early stage of the evolution of this taxonomic lineage, with a duplication event initially separating these genes into two distinct evolutionary groups (clades 1 and 2 in Fig. 4). More recent duplications (e.g., *CcSTE3.1* and *CcSTE3.3* in *C. cinerea* or *LbSTE3.1* and *LbSTE3.3* in *Laccaria bicolor*) (Fig. 4) followed by sequence diversification and recombination (80) are suggested to have led to the high numbers of alleles presently estimated from natural populations of several mushroom species (16, 20, 41, and references therein).

In *C. cinerea* and other Agaricomycetes, each encounter of two monokaryotic hyphae results in promiscuous cell-cell fusion irrespective of whether or not they are compatible at *MAT* (18, 89). However, for the development and maintenance of the dikaryon, nuclei of interacting partners must harbor different allelic versions of genes in at least one sublocus of both *P/R* and *HD* loci (18, 89–91). Classical genetic analyses of these semicompatible interactions in *C. cinerea* and *S. commune* have shown that the *P/R* system is involved in events that follow hyphal fusion, namely in the reciprocal exchange and migration of nuclei and clamp cell fusion (Fig. 5). On the other hand, genes encoded at the *HD* locus repress asexual sporulation (92), regulate pairing of nuclei within the dikaryotic tip cell, and coordinate nuclear division, clamp cell formation, and septation from the subapical cell (1, 18, 93) (Fig. 5). If the two mates share identical alleles at *P/R* and *HD* loci, no nuclear exchange occurs and the fungal individuals resume monokaryotic growth.

Additional pheromone receptor-like genes (i.e., *STE3*-like) have been identified from numerous Agaricomycetes using sequence similarity-based approaches (Fig. 3A), and many fall within one of the three clades of Agaricomycete mating-type receptors (subclades A and B of clade 1 as well as clade 2) (Fig. 4). However, their function remains largely elusive, because reports suggest that these are non-mating-type-specific receptors (94, 95), and experimental evidence from *S. commune* suggests that they are not sufficient *per se* to induce mating-specific development (96). Many of these non-mating-type-specific receptors are located in the close neighborhood of the *P/R* locus, while others are unlinked (Fig. 3A). Three distinctive features of these Ste3-like receptors are seen in comparison to the mating-type-specific receptors: (i) they present longer C-terminal cytoplasmic regions, (ii) lack pheromone genes in close association, and (iii) show lower levels of intraspecific polymorphism. Non-mating-type-specific receptors have been documented in *C. cinerea* (41), *Phanerochaete chrysosporium* (97), *L. bicolor* (98), *Postia placenta* (99), and several species of the order Polyporales (100), and many more can be expected to be identified from whole-genome sequence data. For


Key:
Branch Support values:
< 80
80-94
≥ 95
SH-aLRT
UFBoot
0.5
A
Agaricomycotina
Agaricomycetes
Dacrymycetes
Tremellomycetes
Wallemiomycetes
Ustilaginomycotina
Ustilaginomycetes
Malasseziomycetes
Exobasidiomycetes
Pucciniomycotina
Microbotryomycetes
Cystobasidiomycetes
Pucciniomycetes
Agaricostilbomycetes
B
pheromone
receptor alleles
(or paralogs)
A B
Cryptococcus neoformans JEC20 (AAN75624)*
Kwoniella heveanensis BCC 8398 (ASQB01000005)*,F
Papiliotrema flavescens CF01 CBS 491 (CDR19282)*
Holtermannia corniformis JCM 1743 (00792)***,E
Holtermanniella nyarrowii JCM 11471 (05667)***,C
Filobasidium globisporum JCM 10633 (g1464a)***,A
Filobasidium wieringae JCM11695 (04014)***
Solicoccozyma terricola JCM 24523 (753153)**,C
MATa / A2
Serpula lacrymans S7.3 Slrcb3a (EGN92608)*
Trametes versicolor FP-101664 SS1 TvSTE3.4 (XP_008040861)*
Coprinopsis cinerea Okayama 7#130 CcSTE3.2a (XP_002910431)*
Laccaria bicolor S238N-H82 LbSTE3.2 (632625)**
Coprinopsis cinerea Okayama 7#130 CcSTE3.2b (XP_002910432)*
Serpula lacrymans S7.3 Slrcb3b (EGN92606)*
Dacryopinax primogenitus DJM 731 SSP1 (54701)**,C
Calocera viscosa TUFC12733 (72046)B
Clade 2
Solicoccozyma phenolicus JCM 11743 (02487)***
Solicoccozyma terricola JCM 24518 (02430)***
Filobasidium uniguttulatum JCM 3685 (g994)***,B
Piskurozyma capsuligena JCM 9041 (g128a)***,A
Phaffia rhodozyma CBS 6938 STE3-1 (CED85384)*
Phaffia rhodozyma CBS 6938 STE3-2 (CED85379)*
Cryptococcus neoformans JEC21 (XP_570116)*
Kwoniella heveanensis CBS 569 (ACZ81463)*
Papiliotrema flavescens CF05 CBS 8359 (CDR19326)*
Dacryopinax primogenitus DJM 731 SSP1 (89913)**,C
MATα / A1
Laccaria bicolor S238N-H82 LbSTE3.1 (XP_001888609)*
Coprinopsis cinerea Okayama 7#130 CcSTE3.1 (XP_001834393) *
Schizophyllum commune H4-8 bbr2 (XP_003028243)*
Serpula lacrymans S7.3 Slrcb1 (EGN92607)*
Trametes versicolor FP-101664 SS1 TvSTE3.1 (XP_008040655)*
Subclade A
Trametes versicolor FP-101664 SS1 TvSTE3.2 (XP_008040656)*
Schizophyllum commune H4-8 bar3 (XP_003027970)*
Trametes versicolor FP-101664 SS1 TvSTE3.3 (XP_008040657)*
Laccaria bicolor S238N-H82 LbSTE3.3 (XP_001888610)*
Coprinopsis cinerea Okayama 7#130 CcSTE3.3 (XP_001834401)*
Subclade B
Clade 1
Sporisorium reilianum SRZ2 (CAI59755)*
Ustilago maydis FB2 (AAA99768)*
Moesziomyces antarcticus JCM 10317 (GAK85036)*,E
Ustilago esculenta (ALS87620)*
Ustilago bromivora UB2 (FMVU01000149)*,D
Ustilago hordei ATCC 90510 (AAD56044)*
Ustanciosporium gigantosporum HRK023 (AEY62516)*
Melanotaenium endogenum CBS481 (Z78962)**
Exobasidium vaccinii MPITM (309869)**
Meira nashicola JCM 18503 (01491)***
Exobasidium vaccinii MPITM (243328)*,C
Malassezia japonica JCM 11963 (BCKY01000003)*,A
Malassezia sympodialis ATCC 44340 (AGC13099)*
pra2
Ustilago bromivora UB1 (SAM61874)*
Ustilago hordei 4857-4 (CAJ41875)*
Ustilago xerochloae KVU1000 (AEY62504)*
Moesziomyces aphidis DSM70725 (ETS62588)*,A
Sporisorium reilianum SRZ1 (CAI59749)*
Ustilago maydis FB1 (AAA99766)*
Kalmanozyma brasiliensis GHG001 (EST05906)*,C
Melanopsichium pennsylvanicum 4 (CDI55363)*,C
Farysia acheniorum JCM 8976 (g1680)***,B
Ustanciosporium gigantosporum (AEY62496)*
Anthracocystis flocculosa PF-1 (EPQ30811)*
Microstroma bacarum JCM 3928 (g3732)***,A
Sympodiomycopsis paphiopedili JCM 8318 (g3329)***,B
Meira nashicola JCM 18503 (01493)*,C
Tilletiaria anomala UBC951 (XP_013240297)*
Golubevia pallescens JCM 5230 (06737)***,B
Malassezia sympodialis ATCC 42132 (AGC13097)*
Malassezia globosa CBS 7966 (XP_001731696)*
pra1
Anthracocystis walkeri KVU975 (AEY62525)*
Sporisorium reilianum SRZCXII2 (CAI59763)*
Pseudozyma hubeiensis SY62 (XP_012192235)*
Ustilago esculenta (ALS87613)*
Ustilago xerochloae KVU1000 (AEY62544)*
Ustanciosporium gigantosporum HRK023 (AEY62536)*
Tilletia horrida QB-1 (LAXH01000386)*,D
Tilletia indica DAOM236416 (OAJ04144)*
Golubevia pallescens JCM 5230 (06736)***,B
pra3
Microbotryum lychnidis-dioicae p1A1 (ABU62847)*
Leucosporidium scottii CBS 5931 (CRX79175)*
Sporidiobolus salmonicolor CBS 483 (ADM24772)*
Rhodosporidium toruloides NP11 (XP_016271323)*
STE3.1
Puccinia graminis f. sp. tritici CRL 75-36-700-3 (XP_003338083)*
Puccinia triticina 1-1 BBBD Race 1 (OAV87231)*
Puccinia striiformis f. sp. tritici PST-78 (KNE91469)*
Melampsora laricis-populina 98AG31 (XP_007419438)*
Melampsora laricis-populina 98AG31 (XP_007418988)*
Cronartium quercuum f. sp. fusiforme G11 (54044)**
Cronartium quercuum f. sp. fusiforme G11 (666007)**
STE3.2-2/4
Puccinia graminis f. sp. tritici CRL 75-36-700-3 (XP_003319218.2)*
Puccinia triticina 1-1 BBBD Race 1 (OAV88334)*
Puccinia striiformis f. sp. tritici PST-78 (KNE91511)*
Melampsora laricis-populina 98AG31 (XP_007418099)*
STE3.2-2/3
Puccinia triticina 1-1 BBBD Race 1 (OAV89065)*
Puccinia striiformis f. sp. tritici PST-78 (KNF04269)*
Puccinia graminis f. sp. tritici CRL 75-36-700-3 (XP_003307383)*
Cronartium quercuum f. sp. fusiforme G11 (66280)**
Melampsora laricis-populina 98AG31 (XP_007405301)*
STE3.2-2/1
STE3.2
Kondoa malvinella JCM 3789 (g3896a)***,B
Kondoa subrosea JCM 5735 (g3850)***,B
Cystobasidium pallidum JCM 3780 (00410)***
Erythrobasidium yunnanensis JCM10687 (09100)***
Cystobasidiopsis lactophilus JCM 7595 (06665a)***
Microbotryum lychnidis-dioicae p1A2 (ABU62846)*
Leucosporidium scottii CBS 5930 (LN868509)**
Sporidiobolus salmonicolor CBS 490 (ADM24775)*
Rhodosporidium toruloides ATCC 204091 (EGU11457)*
Saccharomyces cerevisiae S288c (NP_012743)*

example, up to 29 Ste3-like receptors were identified in the genome of *Serpula lacrymans* (Fig. 2). A few theoretical possibilities have been proposed for their function (41). One such possibility considers that they may play a role during monokaryotic fruiting, in a way similar to that described for the *C. neoformans* non-mating-type receptor gene *CPR2*, whose overexpression elicits homothallic reproduction (101). Another hypothesis suggests that these genes may function in vegetative communication within a species or in conspecific recognition avoiding hybridization (41). Experimental studies addressing the function of these non-mating-type-specific receptors are thus critically needed.

Comparative genomics revealed that gene content and organization (synteny) are generally conserved around the *HD* locus in Agaricomycetes. Two genes usually border this locus: one encodes a mitochondrial intermediate peptidase (*MIP*), which has served as a valuable marker for the isolation of *HD* loci from nonmodel species, and the other is known as a beta-flanking gene (*-fg*) for an unknown protein (Fig. 3A) (94, 100, 102). Although this configuration is found in most species analyzed, exceptions have been reported. In *S. commune*, for example, two large rearrangements at the *HD* locus have taken place causing the separation of the *HD* locus into two clearly distinct subloci that now lie more than 500 kb apart and frequently recombine during meiosis (Fig. 3A) (103, 104). A similar situation is observed in *Flammulina velutipes* (winter mushroom or enoki), but the distance between the two *HD* subloci is about 70 kb. Interestingly, whereas synteny analysis of the surrounding regions indicates that the separation of the two *HD* subloci in *S. commune* emerged via transposition of the two gene clusters, the extant arrangement in *F. velutipes* was driven by two large inversions (95). Other exceptions to the typical *MIP–HD1/HD2–-fg* gene arrangement were observed in the cacao pathogen *Moniliophthora roreri*, where both *MIP* and *-fg* are located 40 and 60 kb upstream of *HD1/HD2* (105), respectively, and in the Shiitake mushroom, *Lentinula edodes*, where *MIP* is distant from the *HD* locus (106). Gene order conservation at the *P/R* locus is also observed among Agaricomycetes, although to a lesser extent compared to the *HD* locus. The *P/R* locus in *L. bicolor* was shown to be in a chromosomal region more prone to gene duplication, translocations, and accumulation of transposable elements (98), and in the wood-decay basidiomycete *Botryobasidium botryosum* (order Cantharelalles; Fig. 2), genome inspection revealed the presence of transposons in the immediate vicinity of pheromone and receptor genes (Fig. 3A; this report). Another illustrative example is the position of *STE20* and *STE12* genes, which varies among the species shown in Fig. 3A.

Tetrapolarity with multiple alleles at both *MAT* loci is often suggested to promote outcrossing (107, 108). It is true that tetrapolarity is less favorable to selfing than to outcrossing, because the chances that any two haploid siblings from the same diploid parent are compatible is at most 25% for tetrapolarity, compared to 50% from a bipolar cross (107). However, tetrapolarity also yields only 25% compatibility under outcrossing when both mating-type genes are biallelic, so that tetrapolarity in itself does not favor outcrossing compared to selfing under biallelism. It is multiallelism that may restrict inbreeding, however, and the evolution of high numbers of mating types is suspected to be primarily driven by selection for increased compatibility at syngamy under an already established outcrossing mating system. An evolutionary scenario could be one with ancestral mating-type determinism being biallelic at the *HD* locus and where the high level of gamete dispersal evolves, leading to widespread outcrossing, which in turn selects for rare alleles, yielding multiallelism at the

Figure 4 Phylogeny of Basidiomycota pheromone receptor proteins. Amino acid sequences identified by BLAST from publicly available databases or from genome projects were retrieved for representative species of the tree subphyla of the Basidiomycota. A total of 106 sequences were manually inspected, amended where necessary, and aligned with MAFFT (195), and poorly aligned regions were trimmed with trimAl (196). The phylogenetic tree and branch support were obtained as in Fig. 2, and the tree was rooted with *S. cerevisiae* Ste3p. GenBank accession numbers (*), Joint Genome Institute protein identifiers (**), and RIKEN/NBRP identifiers (***) are given after the strain name, with letters in superscript indicating (a) genomes assembled from available raw sequencing data and inspected locally, (b) genomic contigs/scaffolds lacking gene annotation, and (c) genes whose annotation was corrected. Species highlighted in boldface are shown in Fig. 3, with arrows before their names indicating the allelic version (or paralog) of the pheromone receptor as colored in Fig. 3. Of note, the *STE3.1* and *STE3.2* alleles in the Microbotryomycetes (Pucciniomycotina) displayed the deepest allelic divergence and trans-specific polymorphism, with the *STE3.1* alleles of the different species branching together rather than each of these alleles clustering with the *STE3.2* allele from the same species (42, 45, 137).

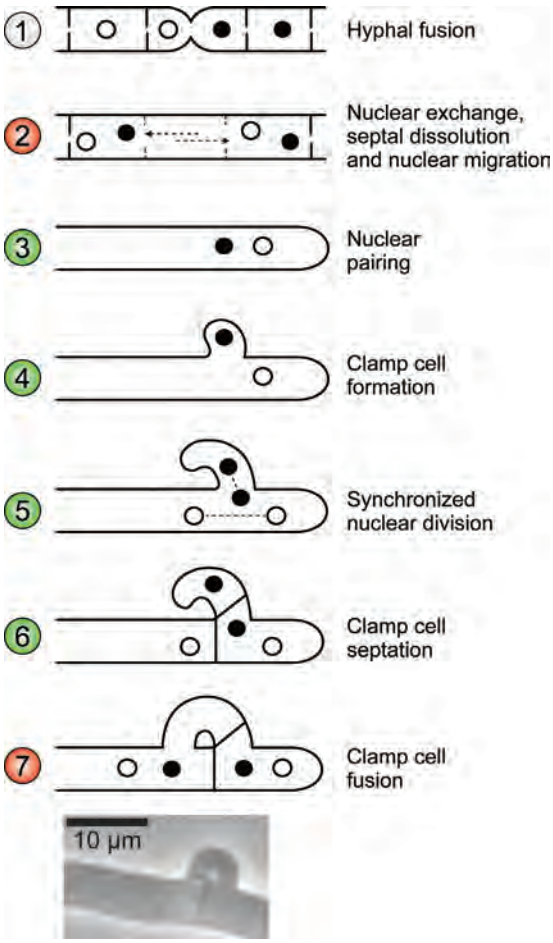


Figure 5 Roles of *P/R* and *HD* genes on the formation and maintenance of the dikaryon in *C. cinerea*. Pheromone signaling is not required to attract mates, and hyphal fusion is mating-type independent (diagram 1). Upon fusion, nuclei enter the mycelium of the other mate and migrate until they reach a hyphal tip cell (diagram 2). During hyphal tip elongation, the two types of haploid nuclei (depicted in white and black, representing different *MAT* genotypes) pair at the tip cell (diagram 3), and at the place where mitosis will take place, a hook-like structure (called a clamp connection) is formed (diagram 4). The two nuclei divide synchronously: one of the nuclei divides in the direction of the clamp cell that is growing backward toward the main hyphae, while the other divides along the main hyphal axis (diagram 5). Septa are generated between the dividing nuclei. This way one nucleus stays temporarily entrapped in the clamp cell, one nucleus of the other type is enclosed in the newly formed subapical cell, and a nucleus of each type is maintained in the emerging hyphal tip cell (diagram 6). The clamp cell fuses with the subapical cell and releases the entrapped nucleus from the clamp cell, restoring the dikaryotic state of the subapical cell (diagram 7). In *C. cinerea*, steps controlled by *P/R* (diagrams 2 and 7) and *HD* (diagrams 3 to 6) genes are colored red and green, respectively. See Casselton et al. (18) and Kües (90) for details. The micrograph at the bottom was obtained from Stajich et al. (86), with permission.

HD locus. Recruiting a biallelic *P/R* locus in mating-type determinism would not favor outcrossing, because again, compatibility odds will be similar under outcrossing compared to selfing. Different scenarios for explaining the incorporation of the *P/R* locus in the mating-type determinism system are possible, such as its role in syngamy or mate attraction, or again, evolution first of multiallelism (e.g., due to population differentiation). Studies addressing the pattern of mating in nature, depending on gamete dispersal rates and polymorphism levels at the mating-type genes, will be fundamental to test some of these predictions.

Bipolar Breeding Systems: Genomic Changes and Evolutionary Considerations

Early studies indicated that, although the tetrapolar mating system predominates in the Basidiomycota, a considerable number of species are bipolar. For example, roughly a quarter of the mushroom species are bipolar (1, 13) and, significantly, they occur phylogenetically interspersed with tetrapolar relatives (100). Owing to the occurrence of multiple bipolar species within the basidiomycetes, the origin of tetrapolarity as the ancestral state to this clade is still debatable. However, several lines of evidence argue strongly in favor of this hypothesis. First, the tetrapolar system is architecturally complex, making it unlikely to have arisen multiple times (23, 109). Second, the same mating-type-determining genes (at both *P/R* and *HD* loci) are involved in mating-type determinism in species of the three major basidiomycete lineages, suggesting a common origin (41, 42, 108). Third, all bipolar species investigated so far display genomic signatures, suggesting that they derived secondarily from a tetrapolar system (detailed below). Based on these observations, the prevailing hypothesis is that the tetrapolar system evolved in a basidiomycete ancestor following the acquisition of a separate locus (the *P/R* locus) for *MAT* determination that segregated independently of the *HD* locus, and with multiallelism evolving in many lineages (17, 41).

As initially envisaged by John Raper (1), and more recently supported by solid molecular evidence (detailed below), bipolarity in the Basidiomycota appears to be the result of simple genetic changes. There are two general ways this can be achieved: (i) by linkage of the *P/R* and *HD* loci into a single, nonrecombining *MAT* region with often only two alleles of each locus being retained or (ii) by loss of function of the *P/R* locus in mating-type specificity, conceivably through mutations that cause constitutive expression of the *P/R*-regulated pathway or that produce a self-compatible allele at the *P/R* locus.

Transition to bipolarity in smuts and *Malassezia* through linkage of *P/R* and *HD* loci

There are now several direct examples that support the formation of a bipolar system through a chromosomal translocation that places both *P/R* and *HD* loci in tight linkage. In the Ustilaginomycotina, a prime example is provided by *Ustilago hordei*, a bipolar smut pathogen closely related to tetrapolar *U. maydis* (Fig. 2) (110). With the molecular analysis of the *MAT* loci, initially in *U. maydis* (26, 59, 111, 112), the genomic basis of the bipolar mating behavior of *U. hordei* was subsequently revealed: both *P/R* and *HD* complexes were physically linked on the same chromosome, 430 and 500 kb apart on the largest chromosome representing the respective *MAT-2* and *MAT-1* loci (Fig. 3B), and the presence of inversions in between these complexes was proposed to cause the almost complete lack of recombination of the complexes (113–115), likely resulting in a subsequent accumulation of transposable elements. Detailed analyses have since then been published on mating types in several Ustilaginomycotina spp.

Complete genomes of five species of closely related genera varying in mating-type organization are available: tetrapolar *U. maydis* (116) and *S. reilianum* (117) and the bipolar species *U. hordei*, with the *HD* and *P/R* loci 430 to 500 kb apart (118), *Sporisorium scitamineum*, with the *HD* and *P/R* loci 59 kb apart (119, 120) (Fig. 3B), and *Ustilago bromivora*, with the *HD* and *P/R* loci 183 kb apart (121). Based on comparison of chromosomal rearrangements between *U. maydis*, *U. hordei*, and *S. reilianum*, it was suggested that the tetrapolar organization in the *S. reilianum* lineage was ancestral and that part of *S. reilianum* chromosome 1 harboring the *HD* locus was translocated to its chromosome 20 harboring the *P/R* locus, to generate the bipolar organization in *U. hordei* (118). This potential scenario was strengthened in the recently published bipolar *S. scitamineum* genome: homologous parts of *S. reilianum* chromosomes 1 and 2 seem rearranged to give chromosome 2 in *S. scitamineum* (120). In all these cases, the presence of many repetitive and transposable elements at the breakpoints suggested they may be functionally involved (122). As stated above, the resulting spacing between the *HD* and *P/R* loci in these species varied between 59 and 500 kb, and this was likely a consequence of increasing numbers of such elements due to decreased recombination.

A similar *MAT* loci configuration has also been recently identified in the human skin commensal yeast *Malassezia* spp. complex that is responsible for dandruff and other skin pathologies. These include *Malassezia sympodialis*, *Malassezia globosa*, and *Malassezia yamatoensis* (123, 124) (Fig. 2). Similar to *U. hordei*, the *P/R* and *HD* loci in these three *Malassezia* spp. are also located on the same chromosome and are separated by large chromosomal regions that are >100 kb in size. Interestingly, comparison of the chromosomal regions encompassing the *P/R* and *HD* loci in these species also suggests that the linkage between the two *MAT* loci are the result of two independent events, with one giving rise to the linkage between the two *MAT* loci in *M. sympodialis* and *M. globosa*, while the other rearrangement led to the *P/R* and *HD* linkage in *M. yamatoensis*. Additionally, analyses of natural isolates of *M. sympodialis* suggest that recombination is still occurring between the *P/R* and *HD* loci in natural populations, indicating that linkage between the two *MAT* loci is not tight. In agreement with this, the 170-kb *MAT* region of *M. globosa* does not contain accumulations of repetitive elements as found at the *MAT-1* locus of *U. hordei* (125), as is expected in regions of suppressed recombination. Moreover, a preliminary examination of the *P/R*-containing region in the genomes of two *M. sympodialis* strains of opposite mating types revealed conserved gene order in the immediate vicinities of the *P/R* gene pair, which are found adjacent but inverted in both mating types (Fig. 3B; this report). The breeding system in *Malassezia* species is thus considered "pseudo-bipolar," a term initially proposed for the breeding system of the red-pigmented yeast *S. salmonicolor* to reflect a situation where multiallelism might occur despite the apparent loose linkage of the two *MAT* regions (45); linkage is expected to be selected for in selfing mating systems, in which rare alleles have no advantage, leading to gradual loss of alleles but two by genetic drift.

Bipolarity in pathogenic *Cryptococcus* spp. complex and in *Trichosporon*

Outside the Ustilaginomycotina, deviations from the classic tetrapolar system have also been observed in a variety of species. For example, all the species within the human pathogenic *Cryptococcus* spp. complex (Tremellomycetes, Agaricomycotina; Fig 2) (126) have bipolar mating systems. In these species, there are two mating types, *MAT***a** and *MAT*α, defined by two alleles (**a** and α) at a single large *MAT* locus (Fig. 3A). The *MAT* locus in pathogenic *Cryptococcus* spp. is ~120 kb in size and contains about 20 genes, including those encoding mating pheromones and their cognate receptors and homeodomain transcription factors (*HD1* or *SXI1* and *HD2* or *SXI2*). There is, however, a distinctive feature regarding the normally two-gene homeo-

domain function in this group of species: the *HD1* (or *SXI1*) gene is unique to *MAT*α strains, whereas *HD2* (or *SXI2*) is specific to the *MAT***a** strains (Fig. 3A). This is in contrast to other basidiomycetes in which a divergently transcribed *HD1/HD2* gene pair is present in each mating type.

Accordingly, each mating type in pathogenic *Cryptococcus* spp. has been reduced to the minimal number of *HD* genes required to function upon fusion of compatible cells (127). Recessive losses of genes from one mating type could likely occur because each is sheltered in a permanent hemizygous state due to recombination suppression within the *MAT* locus, associated with extensive chromosomal rearrangements. However, gene conversion does occur within these regions and at a frequency that is at least comparable to the crossover frequencies in other chromosomal regions (71). Additionally, it has been shown that recombination hotspots associated with the presence of GC-rich motifs are located at the regions that flank the *MAT* locus in *Cryptococcus* (128, 129), which could potentially function as repressors of crossover within the *MAT* locus. Comparison of the *MAT* loci of closely related tetrapolar species such as *C. amylolentus* (71) and *Cryptococcus heveanensis* (70) suggests that the single *MAT* locus in the pathogenic *Cryptococcus* spp. is the result of fusion of ancestral *P/R* and *HD* loci, possibly mediated by repetitive sequences and transposable elements that may act as substrates for nonhomologous recombination (130, 131).

A recent genomic study found that in the oil-accumulating yeast *Trichosporon oleaginosus* (recently renamed *Cutaneotrichosporon oleaginosus*) (Fig. 2 and Fig. 3A), as well as in some of its sister species, the mating-type regions show significant structural differences from those of other Tremellomycetes that possess tetrapolar breeding systems. Specifically, no homologs of the *HD2* transcription factor could be identified in the genomes of *T. oleaginosus* and *Trichosporon asahii*, suggesting that this transcription factor might have been lost during the evolution of the *Trichosporon* spp. complex (132). Alternatively, this gene is absent from only one mating type like the situation in the *Cryptococcus* spp. complex, a possibility which cannot be ruled out since the three sequenced strains carried the same alleles of *P/R* and *HD* genes. However, if the latter hypothesis ends up being true, then such gene loss would represent a case of parallel evolution in *Trichosporon* and the human pathogenic *Cryptococcus* spp. complex given that the last common ancestor of the two lineages most likely contained the standard *HD1/HD2* gene arrangement. It would be of interest in this scenario to test whether the *HD* gene loss is associated with established genetic linkage between the genomic regions that contain the *P/R* and *HD* genes (Fig. 3A). Unfortunately, the fragmented nature of the available genome assembly of *T. oleaginosus* (132) and the lack of descriptions of a sexual cycle for any *Trichosporon* species prevent a more definitive answer at this time.

Expansion of the mating-type locus in *Microbotryum* following linkage of *P/R* and *HD* loci

The findings in the aforementioned bipolar species support the hypothesis of a tendency toward expansion of the regions of recombination suppression in mating-type regions in fungi (133), although it is not yet clear what underlying factors have been driving this trend, e.g., whether it is simply the linkage of the two mating-type loci. In line with this, a more extreme case of the evolution of bipolarity from tetrapolarity by linkage between the two *MAT* loci is observed in the anther-smut fungus *Microbotryum lychnidis-dioicae*. All species belonging to the genus *Microbotryum* are castrating floral smut pathogens that parasitize plants in the Caryophyllaceae family (Fig. 1B), forming a complex of sibling species highly specialized on different plant species (61, 134, 135). The most studied species is *M. lychnidis-dioicae*, which parasitizes *Silene latifolia* (136). This fungal species is bipolar, with only one *MAT* locus and two alleles, called a_1 and a_2 (137). This is among the first fungal species for which bipolar mating types have been described (138) and also the first fungal species for which size-dimorphic mating-type chromosomes were revealed (139). A sex event is required before each new plant infection, and *M. lychnidis-dioicae* displays a highly selfing mating system under heterothallism, performing mostly intratetrad mating (a.k.a. automixis) (140–142). The populations are therefore highly homozygous throughout much of the genome (143, 144).

A recent high-quality assembly of the genome of *M. lychnidis-dioicae* has revealed that bipolarity and mating-type chromosome dimorphism are due to the linkage of the *P/R* and *HD* loci in a very large region of suppressed recombination (145, 146). The centromere is also located in the nonrecombining region (145), which leads to the segregation of mating types in the first meiotic division. The mating-type chromosomes of *M. lychnidis-dioicae* thus do not recombine across 90% of their lengths (a_1, 3.5 mega-base pairs [Mbp]; a_2, 4.0 Mbp), with only small pseudo-autosomal regions at both edges that recombine. The nonrecombining

region is highly rearranged, with at least 210 inversion events. As expected due to the less efficient selection in genomic regions without recombination (147), the cessation of recombination has led to genomic degeneration, with accumulation of transposable elements, nonsynonymous substitutions, biased gene expression, and the hemizygous loss of hundreds of genes (145, 148).

Most other anther-smut fungi are bipolar with dimorphic mating-type chromosomes that also show degeneration (148); one clade has recently been found to have a tetrapolar breeding system: in particular, *Microbotryum saponariae* and *Microbotryum lagerheimii*, which parasitize, respectively, *Saponaria officinalis* and *Silene vulgaris* (73). In these closely related species, the two *MAT* loci segregate independently, being located on different chromosomes, but are linked to their respective centromeres (73). This situation may have resulted from a reversion from bipolarity to tetrapolarity or from the independent evolution of the linkage of *MAT* loci under automixis in the other lineages. Indeed, both bipolarity with linkage to the centromere, as in *M. lychnidis-dioicae*, and linkage of both *MAT* loci to their respective centromeres on different chromosomes, as in *M. saponariae*, are equally favorable under automixis in anther-smut fungi (73, 145).

Transition to bipolarity through loss of function of the *P/R* genes in mating-type determination

It is not surprising that changes in the configuration of the *MAT* loci can alter the mating behavior of individuals, sometimes resulting in transitions between different breeding systems. For example, in tetrapolar heterothallic species, the *HD* locus encodes genes that heterodimerize during mating to form active transcription regulators, ensuring the proper development following syngamy. However, the heterodimer can only form between HD1 and HD2 proteins derived from different alleles. Thus, if recombination occurs between the *HD1* and *HD2* genes resulting in bringing together compatible *HD1* and *HD2* alleles on the same chromosome, a haploid individual bearing this novel *HD* genotype pair would transition from compatibility determined by two sequential compatibility checkpoints (*P/R* and *HD*) to a single mechanism (*P/R*) inherited in a bipolar manner. This newly formed *HD* allele could also be the first step in a transition from heterothallism to homothallism provided that compatible pheromones and receptors are brought together at the *P/R* locus in a subsequent event. Examples of these types of transition have been reported in *C. cinerea*, where fusion of the *HD1* and *HD2* genes from different subunits results in self-compatibility and constitutive sexual development (149). Alternatively, mutations in a pheromone receptor gene in *C. cinerea* resulted in a receptor that was either constitutively activated (150) or that responded erroneously to a normally incompatible pheromone encoded within the same allele (151), removing its function in mating-type determination.

Studies of several other Agaricomycetes, including *Coprinellus disseminatus* (152), *Pholiota microspora* (153, 154), *P. chrysosporium* (94), and *Heterobasidion irregulare* (155), have shown that a transition from a tetrapolar to a bipolar breeding system was indeed due to the uncoupling of the *P/R* from mating-type-determining functions. These bipolar species retained nonetheless a multiallelic *HD* locus, with the number of alleles defining the number of mating types (e.g., ~123 mating types are estimated for *C. disseminatus*) (16, 152). Therefore, a new mating type will arise as soon as a novel *HD* allele is generated. However, evolving novel *HD* alleles that are still able to heterodimerize with extant alleles, while restricting self-activation, will become increasingly challenging (156). Compared to tetrapolar multiallelic species such as *C. cinerea* and *S. commune*, and assuming that *HD* alleles are found at equal frequency in the population, the probability of randomly chosen individuals being compatible in an outcrossing event is theoretically greater in bipolar multiallelic species: only a single locus needs to be compatible, rather than two in the tetrapolar situation (16). Furthermore, considering that mating in mushroom-forming fungi is preceded by basidiospore (meiospore) dispersal, outcrossing rather than selfing is more likely to occur (16, 61). Therefore, and because mating-type compatibility is not discriminated prior to syngamy, the costs of landing on an incompatible mate are in this case very high. It can thus be easily envisaged that mutations at the *P/R* locus causing loss of function in mating-type determination may have been selected for, in some circumstances, because the chance of compatibility would be greatly increased, and there is no selective pressure anymore to restrict inbreeding, which is already avoided by gamete dispersal.

Homothallism in the Basidiomycota

In some fungal species, haploid cells are universally compatible for mating, including among clonemates (a.k.a. intrahaploid mating, same-clone mating, or haploid selfing) (15, 79, 156). We call these species homothallic, and several proximal mechanisms have been proposed to account for such mating behavior: (i) the presence of compatible sets of *MAT* genes, either fused or unlinked, in one haploid genome (primary homothal-

lism), (ii) mating-type switching (either bidirectional or unidirectional), and (iii) the lack of any gene-based mating-type discrimination (also known as the ability for unisexual reproduction or same-sex mating) (reviewed in 157, 158). Some other fungal species, referred to as pseudohomothallic, also have the ability to complete the sexual cycle without the apparent need for syngamy (159–161). However, pseudohomothallism is achieved by packaging two nuclei of compatible mating types derived from the same meiosis into a single spore. This process is thus more similar to diploid selfing via automixis or intratetrad mating and has different genetic consequences compared to intrahaploid mating (79, 162).

Homothallism is present in three main fungal lineages (the phyla Mucoromycota, Ascomycota, and Basidiomycota), indicating that this form of sexual behavior may provide a selective advantage under certain conditions, for instance, by promoting universal compatibility (79, 162). Compared to other phyla, however, homothallic basidiomycete species are less common. Whether this relates to the complex genetic underpinnings of the mating system in this phylum, as described above, or is because they can more easily evolve multiple alleles, allowing high frequencies of mating compatibility, is still an unresolved matter. Primary homothallism has been proposed for a handful of species in the Basidiomycota, most notably *Sistotrema brinkmannii* (163), in which homothallic and bipolar heterothallic individuals are found in the same population, and for the cacao pathogen *Moniliophtora perniciosa* (164). A partial genome sequence is only available for the latter species, which upon examination revealed various genes for putative pheromones and receptors and one or possibly two *HD1* genes (165), but their genomic organization as well as their function during the sexual cycle remain undetermined. We present below details of two basidiomycete species for which there has been progress in understanding the genetic basis of homothallism.

Homothallic breeding system of *Phaffia rhodozyma*

Recently, inspection of the genomes of three strains of the homothallic yeast *P. rhodozyma*, belonging to the order Cystofilobasidiales (Fig. 2) (166), revealed the presence of pheromone precursor and receptor genes organized in two gene clusters, located ~5 kb apart. Each cluster was composed of one pheromone and one pheromone receptor gene (either *MFA1/STE3-1* or *MFA2/STE3-2*; Fig. 3A) (167). In contrast, a single *HD1/HD2* gene pair was found in the genome that was not linked to the *P/R* locus (Fig. 2) (167). In elucidating the molecular interactions involved in mating compatibility in *P. rhodozyma*, David-Palma et al. (168) pursued targeted gene replacement of the *MAT* genes and genetic crosses using single, double, and triple mutants (168). They determined that both *P/R* gene clusters produced intercompatible gene products, enabling self-signaling, and each pheromone only interacted with the receptor of the other cluster (i.e., Mfa1 activates Ste3-2 and Mfa2 activates Ste3-1; Fig. 3A). Additionally, the single pair of HD1 and HD2 homeodomain proteins was also shown to be required and apparently cooperated for normal completion of the sexual cycle (168).

Even though the genetic makeup of *MAT* loci in *P. rhodozyma* is suggestive of primary homothallism, David-Palma et al. (168) pointed out why this configuration departs in several aspects from what would be expected from simply gathering in a haploid genome the intercompatible alleles of *MAT* genes. First, although the two pheromone receptor genes exhibited considerable sequence similarity (~50% amino acid identity), phylogenetic analyses indicated that they were more closely related to each other than to receptor genes in other species, and both seemed to derive from the *MATα/A1*-related allele lineage (Fig. 4). This indicates that the two pheromone receptor genes in *Phaffia* have diverged much more recently than in most basidiomycetes (possibly coalescing within the genus) (M. David-Palma, personal communication), and the extant arrangement may be the result of fusion of two mating-type variants. The second aspect concerns the *HD* genes, in that only one *HD1/HD2* pair is present, but both proteins are required for sexual development. Based on evidence of a very weak interaction between the two *HD* proteins in a yeast two-hybrid assay (168), it was proposed that at the generation of a compatible HD1/HD2 pair within the haploid genome there would need to be one or more mutations relaxing the structural hindrance that normally prevents interactions between HD proteins encoded by the same locus in heterothallic species (168). Similar investigations are currently under way in other species of the Cystofilobasidiales to reconstruct the evolutionary transitions in breeding systems in this lineage (M. David-Palma, personal communication).

Homothallism in *C. neoformans*

While most homothallic species can initiate sexual reproduction through fusion with any other haploid cell, including clonemates, they do so through the presence of two intercompatible mating alleles within a haploid genome. In the human pathogenic fungus *C. neoformans*, the proximal mechanism for homothallism is

different because it concerns haploid cells with a single mating-type allele in their genome (169). Specifically, while *C. neoformans* has a bipolar breeding system in which mating typically occurs between *MAT***a** and *MAT*α cells, it has been found that, in response to nutrient limitation, *MAT*α cells can also complete the sexual cycle in the absence of *MAT***a** cells (169). The α-α diploid state can be established through either (i) cellular and nuclear fusion of two clonemates, (ii) nuclear fusion between mother and daughter nuclei, (iii) endoreplication of the entire genome of a single cell resulting in a diploid nucleus, or (iv) fusion of cells of the same mating type but of different genetic lineages (169). While the first three pathways represent different ways to achieve same-clone mating (haploid selfing), the latter enables outcrossing and thus the possibility to admix genetic diversity despite identical cell-type identity of the mating partners. In all cases, the resulting diploid cell undergoes monokaryotic filamentation and subsequent basidium formation, where meiosis takes place to produce the haploid spores as in the heterothallic mating cycle (Fig. 1C).

Importantly, recent studies using this model basidiomycete have shown that α-α mating between genetically identical cells can generate phenotypic and genotypic diversity *de novo*, including single-nucleotide polymorphisms (less frequently), chromosomal translocations, and aneuploidy (more frequently) which, although usually likely deleterious, may rarely provide a beneficial novelty (170). Moreover, crossovers have also been detected within the *MAT* locus during meiosis of genotypes resulting from α-α mating (129), presumably because the two *MAT*α alleles are colinear, unlike **a**-α combinations (130). It should be pointed out that among the *C. neoformans* natural isolates, the vast majority (up to >99%) are of the α mating type (171). Thus, α-α mating could be beneficial when a compatible mating partner for a heterothallic cross is hard to find in nature. In turn, it is also possible that α-α mating played a role in generating the highly imbalanced mating-type ratio observed among natural isolates. Recently, this type of homothallism determinism has also been identified in the human pathogen *Candida albicans* (172, 173) as well as in the tree wound-infecting fungus *Huntiella moniliformis* (174), suggesting that it could be more prevalent among fungal species than is currently appreciated.

Evolutionary considerations on the costs of homothallism

Although it has been traditionally accepted among mycologists that homothallism evolved to promote same-clone mating (170, 175), it is important to point out that the potential for same-clone mating of a species as assessed in a laboratory setting does not imply that this is the prevalent mating system of the species in nature. Moreover, the sexual cycle involving clonemates does not result in actual recombination, the supposedly main advantage of sexual reproduction (176), while still incurring some costs (e.g., slower reproduction and maintenance of the meiotic machinery). Same-clone mating is less efficient at purging deleterious mutations: homologous chromosomes carrying different deleterious mutations that occurred in a given large clonal lineage can of course be recombined and produce a chromosome free of deleterious mutations (177), but this constitutes more outcrossing (i.e., mating between two different genotypes) than same-clone mating *per se*. Besides, in a population undergoing only same-clone mating, different deleterious mutations that are fixed by genetic drift in distinct clonal lineages cannot be eliminated from the populations, and Muller's ratchet will inexorably lower population fitness (133). Furthermore, same-clone mating cannot bring together different beneficial mutations that evolved in distinct clonal lineages (Hill-Robertson interference). A transition to asexuality would bypass some of the costs of sex while producing haploid progeny that are genetically the same as the participants in same-clone mating.

Homothallism has been suggested instead to have evolved due to the selective advantage of increasing the number of available mates, because it can be seen as a universal compatibility (15, 79). The frequency of outcrossing in homothallic species under experimental conditions of mate choice has rarely been explored, but outcrossing has still been shown to occur in some homothallic fungi (178, 179). Homothallism would therefore be advantageous through universal compatibility under outcrossing. However, given that same-clone mating incurs costs of sex without some of the major benefits of recombination, heterothallism may be favored by selection for increasing the chance to produce recombinant offspring when there is limited haploid dispersal (15, 79). Nevertheless, same-clone mating may allow a benefit from indirect advantages of the sexual cycle without breaking apart beneficial allelic combinations (180, 181). Such advantages include the production of resistant sexual spores as a life history constraint, changes in ploidy level and physiology, virus elimination, and genome defenses that depend on the sexual cycle, such as repeat-induced point mutation (182) or meiotic silencing of unpaired DNA (183). Recent studies also showed that same-clone mating can generate *de novo* mutations and new beneficial phenotypes (170).

New Genome Sequencing Projects: What Can They Tell Us about *MAT* Gene Structure and Evolution?

With the exponential growth of data resulting from several large-scale genomics initiatives, such as the 1000 Fungal Genomes Project at the Joint Genome Institute (184) and the Fungal Genome Initiative at the Broad Institute (185), it is now possible to identify and compare the genomic organization of *MAT* genes across many fungal species. For example, genome surveys allowed the detection of *P/R* and *HD* genes in important plant pathogens with complex life cycles, such as the rust fungi (Pucciniales, Pucciniomycotina; Fig. 3B) (40, 186). Indeed, because many rust species are macrocyclic (i.e., completing their sexual stage on a different and sometimes obscure or unknown alternative host plant) and sometimes unculturable, sexual compatibility has been very difficult to study. Some reports have shed light on possible breeding systems: several *Puccinia* and *Uromyces* spp. may have a simple bipolar system (187), whereas a more complicated tetrapolar system with multiple allelic specificities may be present in *Melampsora lini* (188) and the related oat crown rust pathogen, *Puccinia coronata* (189). Whether these and other species have bi- or multiallelic *MAT* loci remains unknown, but new insight is now arising from different fungal genome sequencing initiatives.

In a recent study, several new wheat rust *Puccinia* spp. genomes were analyzed for the presence of mating-type genes (186). In three cereal-infecting rust fungi (*Puccinia triticina* [*Pt*], *Puccinia graminis* f.sp. *tritici*, and *Puccinia striiformis* f.sp. *tritici*), one divergently transcribed *HD1/HD2* gene pair was found, reminiscent of the organization in other basidiomycetes; two alleles of each were present in the dikaryotic urediniospores, which is the cell type produced upon mating. Preliminary analyses of multiple genome sequences allude to the presence of more than two alleles in nature (G. B., unpublished). Three homologs for the pheromone receptor (Ste3) were identified in the dikaryotic urediniospores. Phylogenetic analysis, comparing Ste3 proteins from various rusts and other basidiomycetes, revealed that one *STE3* gene (*PtSTE3.2-1*) was present in two allelic forms, whereas the other two (*PtSTE3.2-2* and *PtSTE3.2-3*) likely represented the two mating specificities, one in each haplotype genome (186) (Fig. 3B and Fig. 4). This was substantiated by revealing many more single-nucleotide polymorphisms in *STE3.2-1* homologs in *P. triticina*, *P. graminis* f.sp. *tritici*, and *P. striiformis* f.sp. *tritici* among several sequenced genomes; the conclusion, also based on differential expression in various life cycle stages, is that this gene may represent a novel function (186), suggesting that *MAT* pathway components may be repurposed. In further support, a pheromone precursor gene (*PtMFA2*) was identified 650 bp from *PtSTE3.2-2*, divergently transcribed as found, e.g., in the *P/R a2* locus of *U. maydis*. However, the current rather fragmented *Puccinia* genomes did not allow for further conclusions about the possible physical linkage of the *HD* and *P/R* gene complexes to constitute a bipolar arrangement, but based on the current assembly and some mapping data in *P. triticina*, these loci are at least 216 kb apart (186). Current developments of single-molecule sequencing by Pacific Biosciences, focusing on generating very long kb-sized reads, are expected to improve these genome assemblies in the near future.

CONCLUSIONS

The basidiomycete fungi have been found to undertake nearly every permutation of reproductive compatibility mechanisms, and the recent literature reviewed here illustrates that the high diversity at mating compatibility loci has remained for several decades "the most challenging problem in the control of [their] sexuality" (190). The advent of inexpensive molecular tools now provides access to genetic information in a broader range of nonmodel species, including at the population-genomic level, so that more comprehensive databases are being assembled. Such surveys shed light on the generalities of what *does* happen in nature, beyond our glimpses and speculations into what *can* happen in exceptional cases (vis-à-vis reference 191). Just as importantly, the large-scale comparative approaches that derive from improved resolution of phylogenomic techniques (192) may provide new understanding of the ecological and evolutionary forces responsible for transitions in breeding systems and underlying genetic determinants. Fungi thus provide rich opportunities for the match of tractable study systems with questions pertaining to sexual life histories as a defining characteristic retained among diverse eukaryotic lineages.

GLOSSARY OF TERMS

Anisogamy: Situation in a species with two classes of gamete sizes—one class of large gametes (female function) and one class of small gametes (male function); a single genotype can produce both types of gametes in most anisogamous fungi, so that there are no different sexes.

Automixis: A form of diploid selfing resulting from the fusion among products of a single meiosis (i.e., intratetrad mating).

Bipolarity: Breeding system with a single locus controlling mating type; it is often associated with only two alleles in fungi.

Breeding system: Mechanism determining reproductive compatibility, i.e., with no mating type (homothallism) or mating types determined by one locus (bipolar) or with two independent loci (tetrapolar); it is important to distinguish the breeding system from the mating system, because, even if they likely coevolved, the understanding of their relationships and their evolutionary implications can only be precisely achieved by recognizing that they refer to different concepts (15, 17).

Clonemates: Cells with identical genotypes resulting from clonal division of a mother cell.

Diploid selfing: Mating among meiotic products from a single diploid individual.

Haploid selfing (or same-clone mating, intrahaploid mating): Mating among haploid clonemates.

Heterothallism: Situation in which a given haploid genotype can only mate with another haploid genotype carrying a different allele at the mating-type locus (loci). It is important to distinguish the terms "heterothallism" and "self-sterility," the latter usually being used to refer to the inability of diploid selfing rather than haploid selfing in the scientific literature, which have very different evolutionary consequences. Even in fungi, many heterothallic species can undergo selfing (e.g., *Microbotryum* anther smuts; Fig. 1).

Hill-Robertson interference (named after the two scientists who first proposed the concept): A phenomenon referring to less efficient selection because of linkage disequilibria (generated either by drift or in a clonal population); for example, in the case of beneficial mutations appearing in different individuals at two different genes in linkage disequilibrium, selection can only fix one of the two.

Homeodomain protein: Protein that harbors a characteristic approximately 60-amino acid helical protein-folding structure, highly conserved among eukaryotes, that binds DNA at so-called conserved homeobox sequences of about 180 bp and often regulates transcription of other sets of genes; in basidiomycetes, these transcription factors control a set of genes involved in the mating process, including morphological changes. The term "homeo" refers to the term "homeosis designing," the replacement of a body part by another body part in animals, as can occur when transcription factors are affected by mutations.

Homothallism: Situation in which a haploid genotype can mate with any other haploid genotype in the population, including its clonemates; homothallism is typically assessed by the ability of a haploid genotype to produce sexual structures alone in a petri dish; however, it is important to recognize that it does not inform on the mating system actually occurring in natural populations. It is also important to distinguish the terms "homothallism" and "self-fertility," the latter usually being used in the scientific literature to refer to the ability of diploid selfing rather than haploid selfing, which have very different evolutionary consequences. Using "self-fertility" for "homothallism" can only lead to confusion in the actual phenomenon that is referred to and therefore in the understanding of concepts and evolution and breeding and mating systems.

Inbreeding: Mating occurring among related individuals.

Isogamy: Situation in a species in which all gametes have similar sizes.

Karyogamy: Fusion of haploid nuclei following mating.

Mating system: The pairing of mating participants in nature, considering their genetic relatedness, i.e., outcrossing versus inbreeding or selfing (and either haploid or diploid selfing).

Mating types: Different types of individuals or gametes in the population, with gametes of similar sizes, but mating compatibility being usually determined by the exchange of molecular signals and being possible only between different mating types. It is important to distinguish sexes from mating types because the evolutionary causes of their evolution and their evolutionary consequences are very different (15).

Muller's ratchet (proposed by Hermann Joseph Muller): A process in which the chromosomes of asexual populations accumulate inexorably deleterious mutations where the lack of recombination prevents regenerating chromosomes free of deleterious mutation from two homologous chromosomes carrying different deleterious mutations.

Outcrossing: Mating among different genotypes, usually unrelated individuals.

Selfing: Mating among meiotic products from the individual; used in the broader literature to mean "diploid selfing." See Diploid selfing, Haploid selfing.

Sexes: Different types of individuals in the population, one type producing large gametes (females) and the other type producing small gametes (males).

Syngamy: Haploid cell fusion during mating.

Tetrapolarity: Breeding system with two unlinked loci controlling mating type; it is often associated with multiple alleles in fungi.

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Fungal Sex: The Mucoromycota

8

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INTRODUCTION TO THE MUCOROMYCOTA

The Mucoromycota is a newly formalized phylum of fungi that are one of what are sometimes considered the basal lineages in the fungi (1). These species have undergone a different evolutionary trajectory than the Ascomycetes and Basidiomycetes. Generally, the species are difficult to develop into experimental models, but despite this our understanding of mating and sex in the fungi overall has been punctuated with major discoveries being made in this lineage.

In the conventional four-phylum classification, the fungal kingdom included Ascomycota, Basidiomycota, Zygomycota, and Chytridiomycota. The two dikaryon lineages, Ascomycota and Basidiomycota, are monophyletic; on the other hand, the early-diverged and basal fungal lineages Zygomycota and Chytridiomycota (2, 3) were found to be polyphyletic (4, 5). Recent further classification appreciates that within the previous Zygomycota clade, there are two distinct phyla, Mucoromycota and Zoopagomycota (Fig. 1). The class Glomeromycotina—which, judging from the arbuscular mycorrhizal interactions with plants, had been considered an independent phylum—now has been placed within the phylum Mucoromycota, which further includes the classes of Mucoromycotina and Mortierellomycotina (1). The Zoopagomycota include the classes of Entomophthoromycotina, Kickxellomycotina, and Zoopagomycotina. Our current knowledge of sexual development is largely limited to the fungi belonging to the order Mucorales. This chapter, therefore, mainly covers sexual development in Mucorales (13 families, 56 genera, 300 species [6]) (Fig. 2), with a focus on the research from the past decade.

The Mucorales species have been important for a number of reasons, as has been pointed out in previous reviews (7–9). First, it was from research into this group that the first proposal that sex occurred in fungal species was made, for the species *Syzygites megalocarpus* (10). The name "zygospore," hence the common use of the terms "zygomycete" or "Zygomycota," was chosen by de Bary to describe these sexual spores from *S. megalocarpus* (11). Zygospores are the sexual spores formed in these species, and they range up to several millimeters in diameter such that they are visible to the naked eye (Fig. 2). However, higher resolution using scanning electron microscopy reveals that they often have intricate decorations on their surfaces. The second major contribution from the Mucorales was the concepts of heterothallism and homothallism, which were developed based on these species (12). Despite these contributions, mating in these species was not studied as well as it was in the Dikarya, and in some cases was almost forgotten. For instance, the revision of the International Code of Botanical Nomenclature into the International Code of Nomenclature in Melbourne in 2011 aimed to remove the dual naming system for the anamorphic and teleomorphic forms of fungi; however, the new article 59 is specific only to the Ascomycetes and Basidiomycetes. The anamorph of *S. megalocarpus* is named *Sporodinia grandis* (13), so it is ironic that the first teleomorphic fungus still has two names.

STRUCTURE AND GENE CONTENT OF THE *sex/MAT* LOCUS

Sexual reproduction is orchestrated by the mating type (*MAT*) or *sex* locus that is a small region of the fungal genome. Approaches using genetic linkage maps for *Phycomyces blakesleeanus* demonstrated that the sex trait was linked to the *madE* gene and the *lysA* gene (14, 15). This suggested that the sex determination system was encoded by a single, and likely small, part of the genome.

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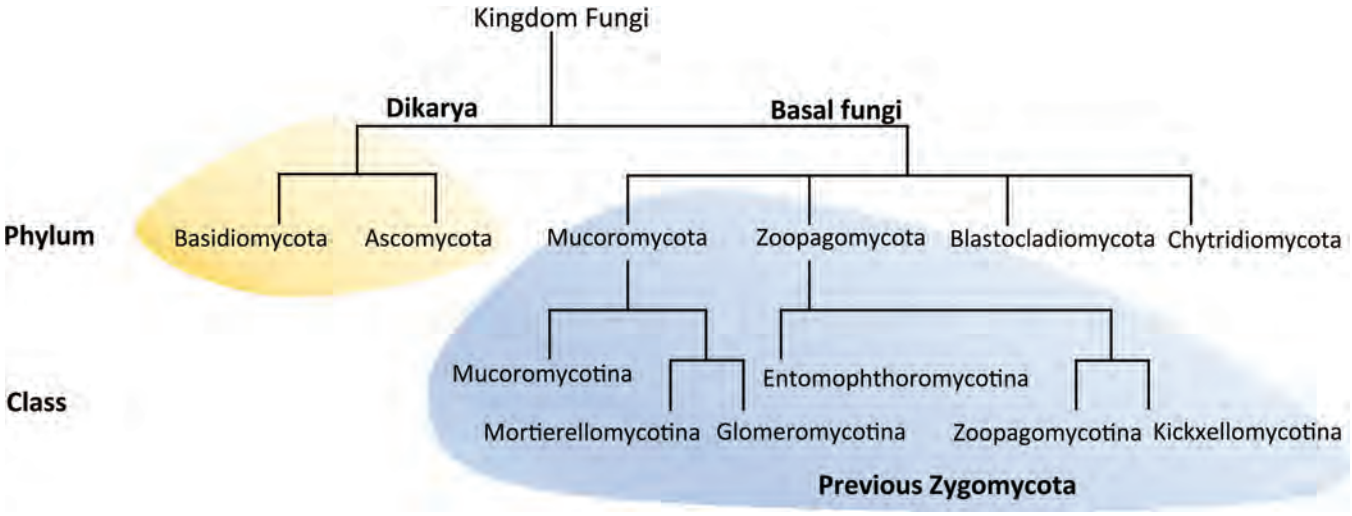


Figure 1 A tree of the fungal kingdom. The previous phylum Zygomycota is polyphyletic and further classified into two phyla, according to Spatafora et al. (1). The phylum Mucoromycota includes the classes Mucoromycotina, Mortierellomycotina, and Glomeromycotina, and the phylum Zoopagomycota includes the classes Entomophthoromycotina, Zoopagomycotina, and Kickxellomycotina.

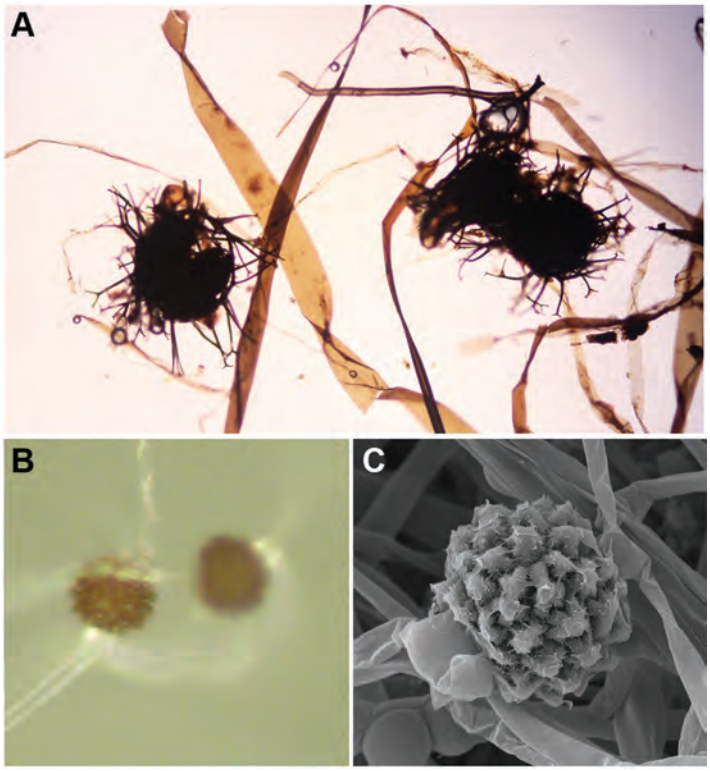


Figure 2 Images of the sexual zygospores produced by species in the order Mucorales. (**A**) Three remnants of zygospores of *P. blakesleeanus* isolated from an environmental sample from Florida, 2014. The long ribbon structures are desiccated asexual sporangiophores. When two mating types of *M. circinelloides* are cocultured in dark conditions, zygospores are formed. (**B**) Light microscopy of two zygospores of *M. circinelloides* and (**C**) scanning electron micrograph of an *M. circinelloides* zygospore.

A seminal study identified the *sex* locus in *P. blakesleeanus* by searching genome sequencing information for possible candidates that may encode either homeodomain or high-mobility group (HMG) types of transcription factors, because these are encoded within the *MAT* loci in the Dikarya (16). The genes discovered encode putative HMG transcription factors, SexP and SexM in (+) and (–) mating types, respectively. The *sexP* and *sexM* genes are idiomorphic, and the sequences of the genes diverge, yet both encode an HMG transcription factor. In both mating types, the *sexP*/*sexM* genes are flanked by conserved genes that encode a putative triose phosphate transporter (TptA) and an RNA helicase (RnhA) (Fig. 3).

Several lines of evidence indicate that this region is a bona fide *sex* locus that represents sexual identity (mating type) and controls sexual development in *P. blakesleeanus* (16). First, any haploid strain contains either the *sexP* or *sexM* gene in the genome, and only mating conditions (cocultures of two mating types) induce the expression of both *sexP* and *sexM* genes and then formation of zygospores. Second, strains containing the *sexP* gene only mate with (–) mating types and strains with the *sexM* gene only mate with (+) mating types, the progeny from crosses with *sexP* or *sexM* segregate 1:1, and no mating occurs between two strains with the same mating type. Third, aneuploid strains that are disomic for the *sexP* and *sexM* genes appear to be self-fertile and produce the initial structures for mating. Finding similar regions that are associated with sexual identity and mating in the genomes of two other Mucorales species, *Mucor circinelloides* and *Rhizopus delemar*, provides additional support that *sexP* and *sexM* are the key regulators of sexual development (17, 18). *P. blakesleeanus* cannot be stably transformed with DNA constructs (19), and thus the roles of *sexM* and *sexP* in mating could not be tested by gene disruption. The disruption of the *sexM* gene in *M. circinelloides* results in loss of fertility in the mutants in mating with (+) mating type cells (and no mating was observed with [–] mating type cells), providing gene functional evidence for the role of the *sex* genes in sexual development in Mucorales (20). Taken together, an arrangement of idiomorphic DNA with either the *sexP* or *sexM* gene, flanked by the *tptA* and *rnhA* genes, clearly represents the *sex* locus in at least three species of Mucorales.

The two *sex* genes differ in sequence and in size between the two mating types; for example, the *sexP* genes of Mucorales are 958 ± 24 bp, and the *sexM* genes are 668 ± 69 bp (the average size of the *sex* genes in *Phycomyces nitens*, *P. blakesleeanus*, *M. circinelloides*, and *Blakeslea trispora*) (21). Currently known *sexM* genes from Mucorales fungi are convergently transcribed with the flanking *rnhA* gene, and the *sexP* genes are also transcribed in a similar way with an exception in the two *Phycomyces* species, where the *sexP* gene is transcribed in an opposite way with the *rnhA* gene (Fig. 3). This observation may indicate that a gene inversion in the *sexM* gene, the *sexP* gene, or both occurred during species evolution in Mucorales. The consensus sequence of a transcription factor binding site, GATCxxxxAxCCAAT, where x is any nucleotide, is observed in the upstream of all known *sexP* genes (21, 22), but what that hypothetical factor might be is unknown.

Although the gene arrangement (*tptA*/*sex*/*rnhA*) associated with the *sex* locus is common in the Mucorales, notable differences have been observed. As mentioned above, the *sexP* gene in *P. blakesleeanus* is divergently transcribed to the *rnhA* gene, unlike other Mucorales, in which the *sexP* genes are convergently transcribed. In addition, the (+) *sex* locus of *P. blakesleeanus* contains a repetitive element in the intergenic space between the *sexP* and *rnhA* genes. The locus in *P. nitens* appears to either have expanded in size or undergone some rearrangement, likely through addition of repetitive element DNA, and it was not possible to join the two *tptA* and *rnhA* regions together by sequencing (21), although they are linked in genetic crosses (A. Idnurm, unpublished data). The (+) *sex* loci of *R. delemar* and *Rhizopus oryzae* additionally contain a gene (*arbA*) encoding a BTB/ankyrin/RCC1 domain protein between the *tptA* and *sexP* genes. Interestingly, in the homothallic species *S. megalocarpus*, the equivalent of the original (–) *sex* locus contains the additional *arbA* gene between *tptA* and *rnhA*. The *S. megalocarpus sex* locus expands to the 5′ part of the *rnhA* gene, while the *rnhA* genes in the other Mucorales species are not part of the idiomorphic *sex* locus. In the *sex* loci of both mating types in *R. oryzae*, *R. delemar*, and *S. megalocarpus*, the direction of transcription of the *tptA* gene is opposite to that of the *tptA* gene in *M. circinelloides*, *B. trispora*, and *Phycomyces* species, suggesting a possibility of another gene inversion in the *tptA* locus during species evolution in Mucorales. Exploring the synteny around the *sex* locus further than *tptA* and *rnhA* reveals additional genes that are conserved between some species. These include the *algL* and *glrA* genes, which encode a predicted alginate lyase and glutathione reductase, respectively. These genes may be valuable additional starting points to identify candidate *sex* loci in the genomes of other Mucorales species.

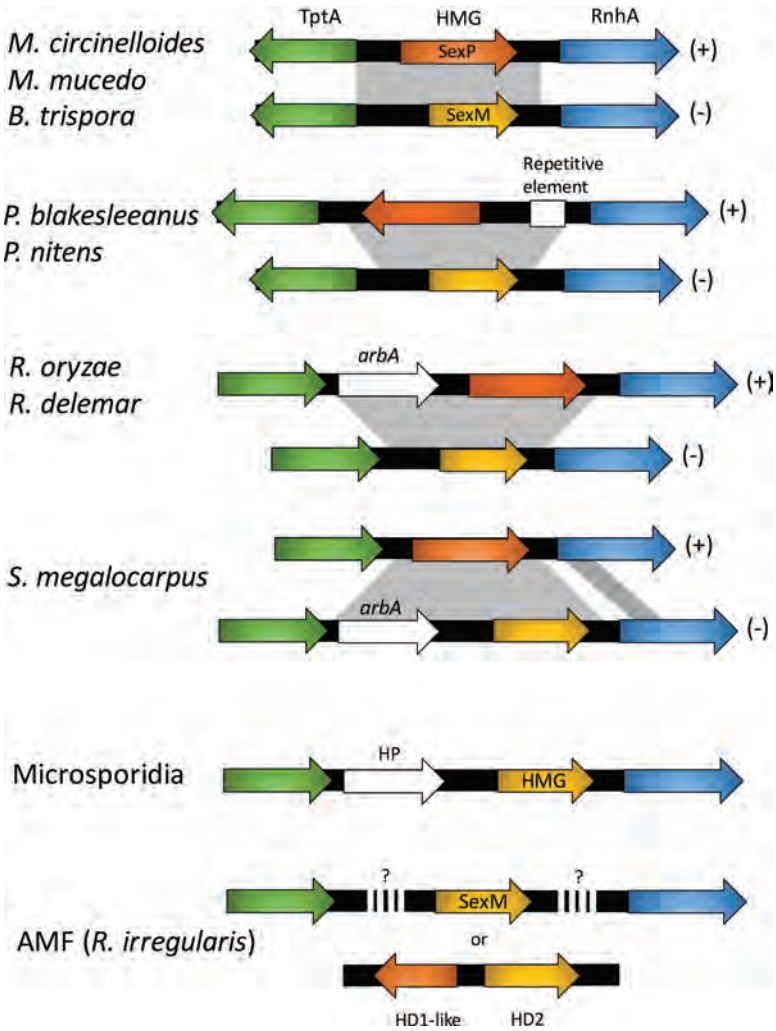


Figure 3 Alignments of genes within or adjacent to the *MAT*/*sex* loci in species of Mucorales, arbuscular mycorrhizal fungi, and microsporidia. Genes encoding allelic or idiomorphic HMG transcription factors, SexP and SexM in (+) and (–) mating types, are flanked by genes encoding a putative triose phosphate transporter (TptA) and RNA helicase (RnhA). This gene cluster is commonly found in the *sex* locus of Mucorales. Although the gene synteny for TptA-SexP/M-RnhA is conserved, notable differences exist. Examples include differences in the direction of the *sexP* and/or *tptA* genes and in the integration of an additional open reading frame or repetitive element, and a level of expansion of the idiomorphic region, which implies expansion of the *sex* locus. Interestingly, microsporidian genomes contain a similar gene cluster for TptA/HMG/RnhA, which might be involved in sexual reproduction of these obligate intracellular eukaryotic pathogens. In the AMF *R. irregularis*, the homologous genes encoding TptA, HMG, and RNA helicase are found, although they are less likely to be linked as is seen in the *sex* locus of Mucorales. In addition, an HD1-like/HD2 gene cluster is encoded in the *R. irregularis* genome, and the two genes are divergently transcribed, which may represent a basidiomycete *MAT* locus-like *sex* locus, with divergently transcribed HD1/HD2 gene pairs.

ROLES OF THE SexM AND SexP HMG DOMAIN PROTEINS IN MATING

How SexM and SexP control the sexual process is currently unknown. Some clues may be available based upon the properties thus far elucidated for the genes and proteins that they encode. The *sexP* gene is expressed during vegetative growth and mating; on the other hand, the *sexM* transcripts are expressed during mating, as observed in *P. blakesleeanus*, *Mucor mucedo*, and *M. circinelloides* (16, 23, 22; S. C. Lee and J. Heitman, unpublished data). Supplementation of trisporic acid to the growing media during vegetative growth also induces the expression of *sexM* (22; Lee and Heitman, unpublished data). Mutation of the *car* genes that are required for the synthesis of the pheromones blocks the induction of the *sexM* and *sexP* genes (23). These observations suggest a connection between the *sex* genes and the pheromone trisporic acid (see below), although the direct connection has yet to be revealed.

There is the potential for an interaction between SexP and SexM to govern sexual development. It is possible that SexP and SexM may control the same set of genes required for mating, for instance, in which the two HMG proteins may form a heterodimer analogous to the homeodomain heterodimers observed in some Dikarya species. However, analysis of the *P. blakesleeanus* proteins in yeast two-hybrid assays failed to find evidence for heterodimerization (23). Alternatively, each SexP or SexM transcription factor may have its own separate target genes that are specific for mating. This would also be consistent with the specialization in functions between the (+) and (–) strains, which are discussed later. Further analysis is needed to define the mode of function of the SexP and SexM proteins.

An HMG transcription factor is also a sole sex determinant in multicellular eukaryotes including humans, in which the Sry HMG factor defines sexual identity (24). This observation supports the hypothesis that the early-diverged group of fungi shares a common ancestor with the metazoans, which is not shared with the Dikarya (2, 25). Mucorales, therefore, might represent an ancestral sexual recognition system that could shed light to help us understand the evolution of sex in metazoans as well as fungi. Indeed, the similarity along with characteristic differences in the *sex* loci in different Mucorales species, including gene inversions, additional gene integrations, and an integration of a repetitive element within the *sex* locus, is in accord with the hypothesis for the earliest steps in the evolution of sex chromosomes posited by Ohno (26).

Heterothallic fungi produce progeny of each mating type in a 1:1 ratio. However, in pathogenic fungi a skew in mating types in isolates from hosts is often observed, suggesting that mating loci may be involved in virulence and pathogenicity. For example, when *Mucor amphibiorum* infection spread among native Australian platypus and nonnative cane toads, an interestingly higher prevalence of (+) mating type was observed (27). The fact that the (+) mating type is more virulent in the toad mucormycosis model than the (–) mating type explains the biased mating types in *M. amphibiorum* isolates (27). Similarly, in the plant pathogenic *Mucor piriformis*, more (–) mating type isolates than (+) mating type isolates were observed in infected plants. Infections of host plants by (–) mating types resulted in larger lesions than did infections by (+) mating types under laboratory conditions (28). In the human pathogenic species *M. circinelloides*, (–) mating type isolates that produce larger spores were found to be more virulent than (+) mating type isolates that produce smaller spores in two different heterologous host models involving larvae of *Galleria mellonella* and zebra fish (20, 29). However, the (–) specific *sexM* mutants did not display a difference in spore size or virulence, suggesting that further investigation is needed to address the connection between mating type, spore size, and virulence in this fungus.

A discovery about the inheritance of organelle genomes and the role of sex determination was made in *P. blakesleeanus* through the analysis of the inheritance of the mitochondrial organelle (30). In *P. blakesleeanus*, strains of both mating type are identical in size and appearance and contribute equal amounts of cytoplasmic material into the equivalent of their gametes (isogamy). Despite this, the organelle genome is only inherited from the (+) mating partner. Other studies that have aimed to link uniparental inheritance of organelles to specific genes on sex chromosomes or mating type loci have been hampered by either large sex determining regions (e.g., the sex chromosomes in humans or the large *MAT* locus in the green alga *Chlamydomonas*) or dissimilar-sized gametes wherein one provides the bulk of the cytoplasm (anisogamy). In the case of *P. blakesleeanus*, the *sex* locus encodes a single gene. In addition, inheritance information from crosses between isogenic strains, identical except for mating type, provided the first definitive evidence that a gene that confers mating type (or gender) also controls cytoplasmic inheritance. Like other aspects of mating in the Mucorales, how mitochondrial inheritance is controlled by SexM or SexP (or both proteins) remains to be elucidated.

A second discovery of sex-specific functions is that the (+) *sex* senses light to inhibit mating, while light has

no effect on the (–) *sex* (23). From these two observations, it appears that the two sexes of *P. blakesleeanus* are specialized in their functions. There has been a long debate over the terms "mating type" or "sex" for fungi, with the argument being made that mating type is more suitable because few differences have been observed between mating types. However, these studies in *P. blakesleeanus* indicate that this argument would be worth reconsidering.

THE EVOLUTION OF HOMOTHALLISM FROM HETEROTHALLIC ANCESTORS VIA REARRANGEMENTS OF THE *sex* LOCUS

A fundamental concept of fungal sexual reproduction is the potential for a single genotype to be able to undergo sexual reproduction by itself versus the requirement of two genotypes, the latter system enforcing outcrossing. Species in which two parents are needed for mating to occur are termed "heterothallic," and those requiring only a single genotype are termed "homothallic." Defining these two mating systems was done with experiments on different members of the Mucorales (12).

One Mucorales species noted for its impact on understanding sexual reproduction in the fungi is *S. megalocarpus*. The mating type loci in this species were sequenced, starting from amplifying fragments with degenerate oligonucleotide primers designed to conserve regions in the *rnhA* and *tptA* genes on either side of *sexM* or *sexP*. Two regions of 20 and 25 kb were sequenced, revealing that this species contains copies of the homologs of both *sexM* and *sexP* within a single hypha (31). The nature of the adjacent genes, i.e., duplicated and then having undergone degradation into pseudogenes, suggests that the mechanism by which homothallism evolved in *S. megalocarpus* was through a duplication event of the *sex* locus in an ancestral heterothallic species. Perhaps most remarkable about the observations made of *S. megalocarpus* is that the equivalent type of arrangement of *MAT* locus genes had been observed to explain homothallism from heterothallic ancestors in ascomycete species, thus providing an example of convergent evolution impacting mating lifestyles in the fungi.

Other Mucorales species are homothallic, but at present the genes required for this process that could potentially explain why they have this reproductive lifestyle have not been characterized. There is an active ZyGoLife project involving the Joint Genome Institute to sequence the genomes of Mucorales species. Future characterization of the *sex* loci in these species should provide additional insight into the connection of evolution between heterothallic and homothallic reproductive strategies.

THE TRISPORIC ACID MATING PHEROMONES

"Sex pheromone" (Greek, *pherein*, meaning to convey, and English, *hormone*) refers to chemicals that are secreted from cells to trigger responses for sexual development in fungi, insects, and even some vertebrates and plants (32–35). In most ascomycetes and basidiomycetes, peptide pheromones, such as α factor or **a** factor mating pheromone for *Saccharomyces cerevisiae* or MF**a**1 or MFα1 pheromone in *Cryptococcus neoformans*, respectively, are involved in sexual recognition between two type cells (36, 37). The Mucorales fungi, unlike the Dikarya species, utilize a volatile organic molecule, trisporic acid, as their sex pheromone (38–40). The synthesis of trisporic acid is initiated from β-carotene in both mating types. To achieve higher amounts of trisporic acid production, both mating types of fungal cells should be in proximity. Sutter et al. revealed that a higher amount of trisporic acid was synthesized by the (+) mating type of *B. trispora* cells in the presence of M-factor that is specifically synthesized by the (–) mating type; similarly, when stimulated by P-factor from (+) mating type cells, (–) mating type cells produce more trisporic acid (41). The authors therefore proposed that the M-factor and P-factor serve as precursors of trisporic acid synthesis (41).

In heterothallic Mucorales, cooperation between two different mating type cells is required to produce trisporic acid (Fig. 4). Cleavage of β-carotene by an oxygenase (42–44) is an initial step in both mating types that results in production of 4-dihydrotrisporin. In the (–) mating type, the resulting 4-dihydrotrisporin is converted into trisporin and trisporol, and these two intermediate compounds need to be delivered into the opposite (+) mating type cells to complete synthesis of trisporic acid. On the other hand, in (+) mating type cells, 4-dihydromethyl trisporate is produced from 4-dihydrotrisporin that results from cleavage of β-carotene. Subsequently, 4-dihydromethyl trisporate is oxidized, producing methyltrisporate (45, 46). It is suggested that the conversion of 4-dihydromethyl trisporate into methyltrisporate is a (–) mating type-specific reaction (40). The enzyme 4-dihydromethyltrisporate dehydrogenase (TDH), encoded by the *tsp1* gene, mediates the conversion of 4-dihydromethyl trisporate into methyltrisporate, and this enzyme activity is differentially regulated in (+) and (–) mating types in *M. mucedo*

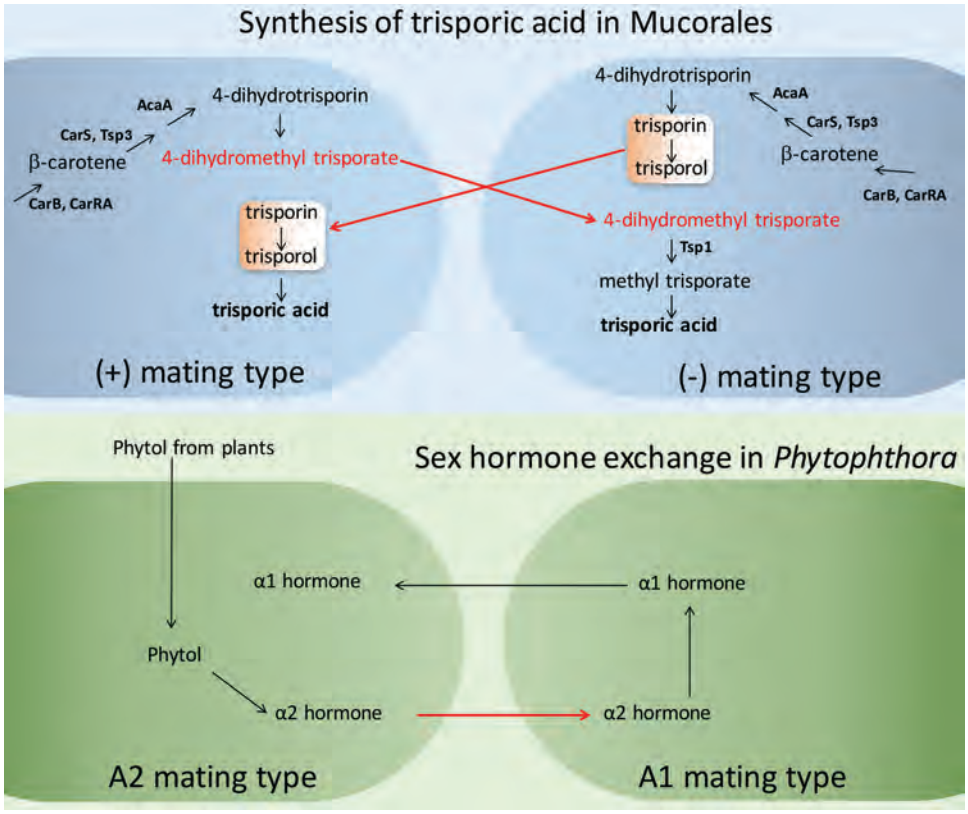


Figure 4 Diagram comparing pheromone processing steps in the Mucorales and the Oomycetes. Synthesis of β-carotene is an important step for production of trisporic acid, and two enzymes, CarB and CarRA, are among the enzymes known to be involved in this process. Synthesis of trisporic acid is initiated by enzymatic cleavage of β-carotene, which involves enzymes such as CarS, Tsp3, and AcaA. Remarkably, the intermediate products after β-carotene cleavage must be exchanged between cells of the two mating types to complete trisporic acid synthesis. For example, the 4-dihydromethyl trisporate in (+) mating type cells is delivered into (–) mating type cells and is converted by Tsp1 into methyl trisporate, and this is then further converted into trisporic acid. Two intermediate compounds, trisporin and trisporol in (–) mating type cells, are transferred into (+) mating cells and are finally converted into trisporic acid. Sex hormone synthesis in the Oomycete *Phytophthora* species is analogous to the inter-mating type collaboration to produce sexual pheromone trisporic acid in Mucorales. The α2 hormone is a sex hormone in the A2 mating type, and it is produced from phytol provided by plants. The α2 hormone, then, must be delivered into A1 mating type cells, where it serves as a precursor of α1 hormone synthesis.

(47). For example, TDH is only activated in sexually stimulated (–) mating type cells but not in nonstimulated (–) or (+) mating type hyphae, indicating that the conversion of 4-dihydromethyl trisporate into methyltrisporate only occurs in (–) mating type cells. Therefore, it is apparent that 4-dihydromethyl trisporate should be transferred from the (+) mating type cells into the (–) mating type cells to complete the synthesis of trisporic acid. Finally, trisporin and trisporol are converted into trisporic acid in (+) mating type cells, and methyltrisporate is converted into trisporic acid in (–) mating type cells.

Interestingly, the intercellular cooperation between two opposite mating types to produce sex pheromones is also observed in the evolutionarily distinct Oomycete lineage (48) (Fig. 4). Heterothallic *Phytophthora* species have two mating types, called A1 and A2, and mating occurs when both mating type cells are cocultured. The A1 mating type produces α1 hormone, and the A2 mating type produces α2 hormone during sexual development (49). The plant hormone phytol triggers the production of α2 hormone in the A2 mating type, and remarkably then the α2 hormone is transferred to A1 mating type cells and eventually converted into α1

hormone (50). Thus, the Mucorales belonging to the fungal kingdom in the Opisthokonts clade exhibit a convergent evolutionary trajectory in the cooperative synthesis of sex pheromone shared with *Phytophthora* species in the Oomycota in the Stramenopiles clade.

P. blakesleeanus mutants that are unable to synthesize β-carotene lack trisporic acid production and exhibit defects in sexual development (51). The β-carotene-deficient mutants, *carB*, *carR*, and *carA*, displayed abnormal sexual development even in unilateral crosses with wild-type opposite mating type cells, where the mating progress is not initiated or arrested in the early stage of zygophore formation without plasmogamy (cell fusion) between two opposite mating type cells (52). *carB* encodes a phytoene dehydrogenase that converts phytoene into lycopene, and *carR* encodes a lycopene cyclase that converts lycopene into β-carotene (53, 54). On the other hand, the *carA* mutant is leaky and has residual phytoene synthase activity that can still generate β-carotene, albeit in reduced amounts, and undergoes complete sexual development when unilaterally crossed with wild type to produce intact zygospores. However, the unilateral cross between *carA* and wild type still displays a biased formation of early sexual structures (52).

Oxidative cleavage of β-carotene is an essential first step to synthesize the pheromone trisporic acid. Gessler et al. demonstrated that degradation of β-carotene by enzymes extracted from the hyphae of *B. trispora* resulted in the production of β-apo-13-carotene. The resulting intermediate induces production of a larger amount of trisporic acid (55). This reaction is mediated by a carotene oxygenase, encoded by the *tsp3* gene, which was first identified in the *R. delemar* genome (25, 56). The *P. blakesleeanus* genome encodes five putative carotene cleavage oxygenase genes, two of which, designated *carS* and *acaA*, were found to be involved in β-carotene catalysis (43). The *carS* gene product cleaves β-carotene into β-apo-12-carotinal, and the AcaA protein further cleaves β-apo-12-carotinal into β-apo-13-carotenone. The *P. blakesleeanus carS* mutant therefore accumulated large amounts of uncatalyzed β-carotene and lacks apocarotenoids, resulting in both bright yellow pigmentation and defects in sexual development (44). The position of these cleavage steps with their origin in the β-carotene molecule is also supported by the isolation of breakdown products from β-carotene of lengths of 7, 15, and 18 carbons in mated cultures of *P. blakesleeanus* (57–59).

The *carRA* gene encodes an enzyme that has a dual activity of phytoene synthase and lycopene cyclase, and the CarRA enzyme is involved in biosynthesis of β-carotene (60, 61). The *B. trispora carRA* gene is constitutively expressed in the (+) mating type; however, the level of *carRA* mRNA is increased in the (–) mating type as vegetative growth progresses (160-fold increase at 144-hour pi). The *carRA* gene is also upregulated at an early stage of sexual development (25-fold at 48-hour coculture of both mating types); however, the expression of the gene is no longer elevated as coculture progresses. Thus, at the 144-hour point, in a (–) mating type mono-culture, the expression of the *carRA* gene is even higher than in (+) and (–) mating type coculture, indicating an even higher accumulation of β-carotene during vegetative growth.

The enzyme TDH is activated only in sexually stimulated (–) mating type hyphae, although the *tsp1* gene encoding TDH is constitutively expressed regardless of mating type and sexual or asexual development in *M. mucedo* (47). The regulation of TDH activity remains unknown; additional complexities include the presence of multiple copies in the genomes of some Mucorales and predicted differences in substrate binding sites and capabilities of dimerization (62). In *B. trispora*, the *tsp1* gene is downregulated as mating progresses, and there is no difference in the level of expression of the gene between mating and vegetative conditions (63). However, the *tsp3* gene, which encodes the carotenoid cleavage dioxygenase and is involved in the initial cleavage of β-carotene, is highly expressed, and its expression remains elevated during the coculture of two mating type cells of *B. trispora*. During vegetative growth, supplementation of the intermediates during production of trisporic acid from β-carotene induces the higher expression of *tsp3* in the (+) mating type compared to in the (–) mating type. Interestingly, there were differences in β-carotene production in the two Mucorales species, *B. trispora* and *M. mucedo*, in response to the supplementation of trisporic acid and its intermediates, indicating that species-specific regulation of trisporic synthesis likely exists (63).

As mentioned above, there is convergent evolution in terms of pheromone processing between the Mucorales and Oomycetes. More broadly, the processing of the pheromones in the Mucorales shares some striking similarities to the processing of the Dikarya peptide-based mating pheromones. Both chemicals are synthesized as longer molecules and then processed and modified multiple times to produce the mature product. However, the chemicals themselves are different, and it is also interesting that the first two steps for processing from β-carotene, at least in some Mucorales, appear to be a result of likely subfunctionalization of the carotenoid oxygenase after the genome duplication reported in

some species (64). The effect of this is that one copy in *P. blakesleeanus*, CarS, recognizes β-carotene to synthesize β-apo-12-carotinal. The second copy, AcaA, has no activity on β-carotene and instead cleaves β-apo-12-carotinal as its substrate.

MENDELIAN AND CLASSICAL GENETICS

The first genetic map in the Mucorales was produced in *P. blakesleeanus*. In this species, the isolation of mutants was made by chemical means, and through the painstaking method of first crossing the mutation into the opposite mating type, and then combinations of crosses were performed to measure genetic linkages based on the phenotypes of mutants (14, 15). This genetic analysis was able to generate a map with 29 genes and the positions of 11 centromeres.

More recently, another genetic map was made that used molecular markers, based on PCR restriction fragment length polymorphisms, in 121 progeny from a cross between two wild-type parents (65). This 1,583-centiMorgan map generated between 9 and 12 linkage groups (depending on the stringency of cut-off for linkage between the markers) and an estimated 33 kb between centiMorgans. As part of the mapping process, a number of genes known from classical mutagenesis were predicted and point mutations identified in the corresponding mutants. This map provided physical linkage for genetic markers onto the genome sequence and indicated strong congruence between the original mutant phenotype segregation and molecular marker maps. It also revealed regions in which the current genome sequencing assembly could potentially be refined in the future.

In the absence of reliable systems for genetic transformation in *P. blakesleeanus* (19), positional cloning has been the only option to identify genes. In these cases, mutations were induced by chemicals such as nitrosoguanidine.

In the first reported example of positional cloning that used molecular markers spaced across the genome, the genetic basis behind the *madI* mutation causing reduced phototropism was identified. Unknowingly, this was a difficult genetics task because the *madI* strains turned out to be heterozygous wild-type/*madA* mutants (64). MadA is a blue-light sensor homologous to the white collar 1 protein of *Neurospora crassa* (66). Hence, the segregation analysis could only poorly define the best linkage for *madI* within the genome to markers near *madA*. It is not clear why the *madI* strains are maintained as wild-type/*madA* heterozygotes. One hypothesis is that the strains are heterokaryons, with two nuclei that each have a deleterious mutation such that in isolation the strain would not survive, but in the heterokaryon there is cross-complementation of these mutations.

Similarly, the *madC* gene that is required for phototropism has been identified by positional cloning (67). A genetic linkage between the genes for carotene biosynthesis and *madC* had first been made based on analysis of just seven zygospores (68). Positional cloning linked the *madC* mutations to markers near three genes impacting carotene synthesis, and the *madC* gene was identified as carrying loss-of-function mutations in a putative Ras GTPase activating protein. How this gene or Ras impacts phototropism is unknown; however, mutation of the homolog in the ascomycete *N. crassa* defined a role for this gene in the circadian banding of the asexual spores (67).

POPULATION GENETICS

Little work has been performed to understand the population structures of members of the Mucorales (reviewed in 69). This is an important area for future investigation, for instance, in the context of the potential population skews in distribution of mating types seen in pathogenic members. Work with *Phycomyces* species has demonstrated that both mating types coinhabit some locations (21) and that zygospores can be isolated from the environment, indicating that mating occurs in nature (Fig. 2A). Curiously, a sample from one location in Michigan in 2012 contained both *P. blakesleeanus* and *P. nitens*, i.e., a sympatric distribution (21). Given that members of the Mucorales are often found in similar locations (e.g., dung of animals) and use similar sex pheromones, how Mucorales species compete for similar resources also warrants future investigation.

SIMILARITIES AND DIFFERENCES BETWEEN THE MUCORALES AND OTHER BASAL FUNGI

Microsporidia are obligate intracellular pathogens, and their hosts range from single-celled protists to nematodes to humans (70). Microsporidia were once considered early-diverged eukaryotes and are now considered to be a sister group of fungi (71). Interestingly, their genomes contain a locus that exhibits a high similarity to the *sex* locus of Mucorales (18) (see Fig. 3). Two human pathogenic microsporidia, *Encephalitozoon cuniculi* and *Enterocytozoon bieneusi*, have a microsynteny of genes encoding TptA, an HMG-domain protein, and

RNA helicase in their genomes. An additional open reading frame for a hypothetical protein exists in the intergenic areas between the genes for TptA and the HMG-domain protein in both microsporidial species. Some other microsporidia species also share loci with a similar architecture in their genomes. The gene cluster of *tptA/sex/rnhA* is unique to the Mucorales and microsporidian lineages; i.e., the cluster has not been observed in any additional branch of fungi. Microsporidian genomes retain genes that function in meiosis, including the core components needed for meiosis (18, 72). Therefore, these observations suggest that microsporidia evolved from ancestral sexual basal fungi and may retain extant sexual cycles (73).

Heterozygous diploids of microsporidia may be indicative of extant sexual cycles. An *E. cuniculi* genome survey found that multiple loci exhibit heterozygosity, indicating that the species is diploid and may undergo sexual development (74–76). *E. bieneusi* and *Vairimorpha* species show alternation of karyotype during the infection cycle from the diplokaryotic phase to monokaryotic, which might be associated with sexual cycle (77, 78). A few microsporidian species that exhibit a transition between monokaryotic to diplokaryotic phases display evidence of gametogenesis and syngamy (79–81). For example, uninucleate mosquito microsporidia infect the host cells and then undergo repeated binary fissions to form enlarged multinucleated plasmodia. The plasmodia then break into uninucleate cells, which is called gametogony and is analogous to gametogenesis (80). A similar infection cycle was also observed in the ant microsporidian *Kneallhazia solenopsae*. However, a bona fide sexual cycle in microsporidia has not been observed in nature or under laboratory conditions, and associations of the *sex*-like locus in mating need to be further investigated.

The Glomeromycota is another basal fungal lineage and has been considered an independent phylum but was most recently placed within the new Mucoromycota phylum (1). The arbuscular mycorrhizal fungi (AMF) in this clade have been considered ancient asexual fungi. This long-standing view has been challenged by evidence of recombination in natural populations of AMF and conservation of meiotic machinery in the AMF genomes (82), which might imply ongoing sexual reproduction (83–85). A recent study by Riley et al. revealed that there are significant numbers of mating-type-A (MATA)-HMG homologs in the genome of *Glomus intraradices* (*Rhizophagus irregularis*) (86). In *R. irregularis*, the genes encoding homologs of TptA, SexM, and RNA helicase are not likely syntenic as seen in Mucorales and microsporidia (87) (see Fig. 3). In the Mucorales and metazoans, HMG proteins such as SexP/M and Sry, respectively, are key sex determinants. In some basidiomycetes, HMGs are not part of the mating locus; however, they are major regulators of sexual reproduction, and examples include Rop1 and Prf1 in *Ustilago maydis*, Pcc1 in *Coprinus cinereus*, and Mat2 in *C. neoformans* (88–90). It is therefore possible that some of the MATA-HMG encoded by the AMF may play roles in sexual development.

However, another study recently proposed that a pair of divergently transcribed homeodomain (HD) genes may represent a locus that governs sexual cycle in *R. irregularis* (91). The authors revealed that the fungus encodes HD1-like and HD2 proteins in a single locus and that sequences of the locus vary among 24 *Rhizophagus* species isolates (see Fig. 3). The syntenic HD1/HD2 gene locus represents a *MAT* locus in some basidiomycetes, and the sequences of the loci vary based on mating types. Thus, the diverged HD1-like/HD2 locus can be a *MAT* locus, and there are extant sexual cycles in *R. irregularis*. If the HD1-like/HD2 is the *MAT* locus in Glomeromycota, the hypothesis that HMG protein is an ancestral sex determinant in the fungal kingdom is challenged. An alternative hypothesis can be proposed that both HMG and HD1/HD2 are ancestral sex determinants and that during evolution of the fungal kingdom, a random loss of one or the other occurred. There is no direct evidence that the AMF's HD1-like/HD2 is the *MAT* locus. Therefore, it is as yet premature to support one hypothesis over the other. Further investigations incorporating more basal fungal lineages would provide robust information to elucidate the evolution of the *sex/MAT* locus in fungi.

CONCLUSIONS AND AREAS FOR FUTURE RESEARCH

A decade ago we reviewed the knowns and unknowns of the mating systems in fungi outside of the Dikarya lineage (7). Since then, some of the mysteries surrounding sexual reproduction in at least the Mucorales species have been resolved, including the discoveries of the genes within the mating type (*sex*) locus and some of the genes controlling pheromone biosynthesis. We would argue that the Mucorales have once again contributed to our understanding of reproductive systems in the fungi. However, it is worth reflecting that the same time separates the evolution of mating type systems in the Mucoromycota and the Dikarya lineage as other early fungal lineages. Mating systems in these other lineages still remain to be determined. The ambiguity of the possibility of a *MAT/sex* locus in the

Glomeromycota could be better clarified with information on other lineages. How well conserved is the pheromone system or the HMG-domain determinants of sex type, as seen in the Mucorales in the genera *Phycomyces*, *Mucor*, and *Rhizopus*? For instance, species in the genus *Mortierella* have been proposed to use the same pheromones as the Mucorales (92), yet the genome sequences that are available for this genus lack the genes for synthesis of β-carotene. The possibility of there being a conserved and common *sex* locus in all of the Mucorales is also not yet clear: candidate loci are present in the draft genomes of many of these species, including the earliest Umbelopsidaceae lineage, yet clear *sex* loci are absent from some genome sequences (93). A better set of genome sequences in these fungi based on current knowledge of their evolution (94) holds the promise of exciting new discoveries.

GLOSSARY

Anamorphic: A growth form in fungi that features only asexual reproductive structures (*cf*. teleomorphic).

CentiMorgan: A unit of genetic distance between markers, which may be genes, traits, or molecules. One CentiMorgan represents one recombination event per 100 progeny between two markers. The term honors the American geneticist Thomas Hunt Morgan.

Dikarya: A subkingdom within the fungi that includes the Ascomycota and Basidiomycota phyla. The name derives from stages in the life cycle of species that have two haploid nuclei per cell or cellular compartment prior to their fusion to initiate the meiotic cycle.

Gametogenesis: The formation of gametes, which are haploid cells dedicated to fusion during the sexual cycle.

Gametogony: In the microsporidia, a process in which a cell containing multiple nuclei divides into cells that can be used as gametes.

Genetic linkage: A state in which one or more genes or other markers, DNA polymorphisms or phenotypic, are close to each other based on genetic crosses, implying physical proximity on the same chromosome.

Heterokaryon: A hypha or other cell type in which more than one type of nucleus is present.

Heterothallism: A sexual reproductive system that requires the contribution of two different isolates of fungi (*cf*. homothallism).

Heterozygosity: A situation in which an organism contains two alleles (copies) of a gene that are different in sequence.

High-mobility group (HMG): A domain within proteins that binds to DNA. HMG-domain proteins are involved in conferring the sex or mating type in animals and some fungi.

Homeodomain: A domain within proteins that binds to DNA. Homeodomain proteins are involved in conferring the mating type in some fungi.

Homothallism: A sexual reproductive system requiring only a single isolate of fungus (*cf*. heterothallism).

Idiomorphic: Regions of DNA that do not share sequence similarity yet occupy the same site in the genome.

***MAT* locus/*sex* locus**: A region or regions within a fungal genome that are required for the sexual process. To date, these all include transcription factors with homeodomain or HMG domains, and in some cases in basidiomycete species, genes for pheromone synthesis and pheromone receptors.

Physical linkage: The presence of genes or other genetic elements on the same piece of DNA.

Plasmodia: A growth form in which the cytoplasm contains multiple nuclei.

Plasmogamy: The step in mating in fungi where fusion between two parents results in a mixture of cytoplasmic material, but prior to fusion of the nuclei.

Syngamy: The fusion of two cells.

Teleomorphic: Referring to a growth form in fungi that features sexual reproduction (*cf*. anamorphic).

Trisporic acid: An 18-carbon terpenoid molecule that is formed during the production of pheromones in members of the Mucoromycota.

Zygophore: A specialized hypha produced by Mucorales species that initiates fusion for the formation of the zygospores; equivalent to a gamete.

Zygospore: The sexual spore produced by species in the phylum Mucoromycota.

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Sex and the Imperfect Fungi

9

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INTRODUCTION

Sexual reproduction is a ubiquitous feature of the eukaryotic kingdom with the many benefits of sex in generating genetic diversity as substrates for evolutionary selection being well known. When two different partners come together, there is the generation of genetic variation in the offspring, through the processes of crossover and recombination during meiosis, enabling response of future generations to environmental selection pressures (1–4). Sexual reproduction also allows the repair of random epigenetic or conventional genetic damage by recombination with homologous chromosomes and can mask lethal mutations (4, 5). In addition, sexual recombination alleviates clonal interference and prevents deleterious mutations hitchhiking to fixation (6). Indeed, there are so many benefits to sexual reproduction that exceptions that are purely asexual have been termed "evolutionary scandals" (7). As a result, supposed ancient asexual species such as the bdelloid rotifers (an exclusively female class of over 460 rotifer species thought to date back several million years) and darwinulid ostracods (a family of around 30 crustacean species thought to have been exclusively female and asexual for over 200 million years, but for which very rare living males have recently been described) have gained notoriety (8–10). It therefore comes as a great surprise that, until recently, approximately 20% of all fungal species were considered to reproduce only by asexual means, with no recognized sexual cycle, based on knowledge of described fungal species (11, 12). Indeed, in some phylogenetic groupings such as the Ascomycotina, up to 40% of taxa surveyed were deemed to be asexual (13). This is despite the fact that sexual reproduction in fungi can have additional benefits such as the production of fruit bodies and sexual spores that are resistant to adverse environmental conditions, thereby promoting survival of sexual offspring; it can provide a transient diploid arena for selection of genes; and sex can favorably impact genome evolution (14–17). Asexual species are also proposed to be short-lived evolutionary "dead ends" subject to rapid extinction (12, 18). Fungal species lacking a known sexual cycle have been referred to as "imperfect" or "mitosporic" fungi and have been grouped into the "Fungi Imperfecti" or "Deuteromycota," although phylogenetic analysis has shown that these are artificial groupings not based on taxonomic relationship (19). The terms "asexual" and "imperfect" are used synonymously in this review.

Therefore, the imperfect fungi appear to present an evolutionary paradox. However, in the past few decades, there has been accumulating evidence that many of these supposedly asexual species might actually have the potential for sexual reproduction and thus exhibit what has been termed "cryptic" (20, 21), "clandestine" (22), or "covert" (23) sexuality, i.e., having the presence of a sexual cycle that has not yet been directly observed. There are various lines of evidence to support this assertion such as analysis of population genetic markers and the recent, fast-growing number of sequencing data sets from asexual yeasts and filamentous fungi indicating that they harbor mating-type loci, and thus have the potential for a sexual life cycle (see further details below). Indeed, the suggestion of cryptic sexuality has been fulfilled or "consummated" recently for several previously considered asexual fungal species when mating and sexual cycles were conclusively demonstrated for the first time. Prominent examples include the discovery of mating in the opportunistic yeast pathogen *Candida albicans* (24, 25), and both mating and complete sexual cycles in the opportunistic filamentous fungal pathogens *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus parasiticus* (26–28), as well as the biotechnologically relevant species *Penicillium*

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chrysogenum, *Penicillium roqueforti*, and *Trichoderma reesei* (29–32; S. S. Swilaiman, H. Darbyshir, J. Houbraken, R. Samson, and P. S. Dyer, unpublished results).

This article will review how these recent advances were made. It will also speculate on the evolution of asexuality in fungi, including consideration of whether there are any truly imperfect fungi, what characteristics they might have, and the factors that could favor their appearance. Special attention is given to the ascomycete group of fungi from which most discoveries have been made.

EVIDENCE FOR SEXUAL POTENTIAL

Various complementary approaches or "sex tests" (33) have been used to provide evidence of the potential for sexual reproduction in asexual species as will now be described.

Population Genetics

One of the first indirect lines of evidence for sexual recombination in supposed asexual species came from population genetic studies. It would be expected that the genetic diversity, both in terms of genotype and phenotype, is higher in sexually propagating populations than in exclusively asexually propagating clonal species. Various molecular and physiological markers have been used in such population genetic analyses. For example, sequencing with arbitrary primer pairs (SWAPP) provides a molecular method to find and characterize genetic markers with nucleotide-level resolution. In the human pathogen *Coccidioides immitis* this technique provided molecular evidence for recombination in a fungus for which no sexual stage has so far been described (34). Alternatively, the distribution of alleles of unlinked loci or microsatellite markers has been analyzed in field populations. It would be expected that if a species were freely sexually recombining, then this would result in most unlinked loci being associated randomly in populations, whereas if there were a predominantly clonal and asexual reproduction, then nonrandom association of alleles ("linkage disequilibrium") would be observed. Thus, evidence of recombination through random association can be taken as an indication of a sexual life cycle. For example, a total of 236 isolates from *Diplodia pinea*, a haploid opportunistic plant pathogen, were characterized using 13 microsatellite markers. Analysis of these markers confirmed previous results that *D. pinea* has a high level of gene and genotypic diversity (35). In parallel, sequencing of seven molecular markers from 31 isolates of the animal-associated asexual *Penicillium dipodomyis* provided evidence of genetic diversity and recombination suggesting cryptic promiscuity (36). In previous work, evidence of recombination had also been observed in populations of *A. flavus*, *A. parasiticus*, and *A. fumigatus* based on DNA fingerprinting and the large number of vegetative compatibility groups present within populations (reviewed in reference 37), and in *P. chrysogenum* based on microsatellite analysis (38)—all species that were, at the time, considered purely asexual. However, it is cautioned that there are possibly other explanations for genetic diversity in populations such as parasexual reproduction (see Daskalov et al. [176]).

Mating-Type Genes

Mating-type (*MAT*) genes have provided fundamental insights into the reproductive status of asexual fungi through studies both of their distribution and also functional activity. By way of background, two main types of fungal sexual breeding systems can be distinguished: (i) heterothallism, involving two individuals of opposing mating type, and (ii) homothallism, which refers to sexual reproduction by selfing of an individual strain. Heterothallic sex maintains genetic variation due to mixing of genotypes, whereas homothallic sex can lead to clonality, similar to that observed in asexual reproduction (12). In heterothallic species, sexual recombination occurs between morphologically identical partners containing opposite *MAT* loci, which, in the case of ascomycetes, consist of dissimilar sequences occupying the same locus on the chromosome. In contrast, homothallic species normally contain genes for both *MAT* loci, which can either be located at a single *MAT* locus or on different chromosomes (21, 39). In most fungi, the mating-type locus represents a relatively small region of the genome, less than a few thousand base pairs, expressing transcription factors that determine both the haploid cell type specificity and/or the diploid zygote fate. Only very few fungi possess larger dimorphic sex chromosomes analogous to those of animals (reviewed by Heitman [40]).

For the most part, our general understanding of mating-related processes in ascomycetes is based on knowledge obtained from studies with the hemiascomycete baker's yeast *Saccharomyces cerevisiae* and the filamentous euascomycete (Pezizomycotina) *Neurospora crassa* (39). In *S. cerevisiae*, two alternative mating-type (*MAT*) loci, namely *MAT*α and *MAT***a**, determine the sex of individual strains (41). These loci are termed idiomorphs to indicate that they do not represent alleles of a single gene (42). In addition, each yeast strain carries silent mating-type loci of both

*MAT*α and *MAT***a**, thus allowing infrequent switching of the mating-type identity. In *N. crassa*, *MAT* loci are designated *mata* and *matA*, whereas in most other euascomycetes the terms *MAT1-1* and *MAT1-2* are used (21, 43–45). *MAT* loci from euascomycetes harbor one or more open reading frames (ORFs), of which at least one codes for a *MAT* transcription factor (TF) (21, 46). In contrast to yeasts, euascomycetes normally have only one active mating-type locus, with no extra silent mating-type loci. *MAT* loci have been characterized from a broad range of homo- and heterothallic euascomycetes (21, 39, 43, 47). Mating-type loci in sordariomycetes seem to be more complex with more than one open reading frame per mating-type locus. Heterothallic members of the Eurotiales are usually less complex and normally contain only a single functional open reading frame as depicted for various *Aspergillus* and *Penicillium* species in Fig. 1. This latter group can be considered to provide ideal experimental systems for gaining mechanistic and functional insights into *MAT* TFs and their various cellular functions as will be focused on below (48–51).

As a rule, the *MAT1-1* locus encodes an α-domain TF, and the alternative idiomorph, *MAT1-2*, is characterized by a gene coding for a TF with a high-mobility group (HMG) domain. The corresponding genes are generally referred to as *MAT1-1-1* and *MAT1-2-1* (45, 47). While DNA-binding HMG proteins are ubiquitous and found in a wide range of eukaryotes, α1-domain proteins seem to be limited to the fungal kingdom. The prototype of an α-domain protein is Matα1p from *S. cerevisiae*, whose function has been studied by genetic dissection (52). From this study the α domain was shown to act as a degradation signal for a pathway defined by the SUMO-targeted ligase Slx5–Slx8, which suggested a coordinated regulation in the turnover of regulatory transcription factors to ensure rapid mating-type switch in yeast. Extensive sequence comparisons,

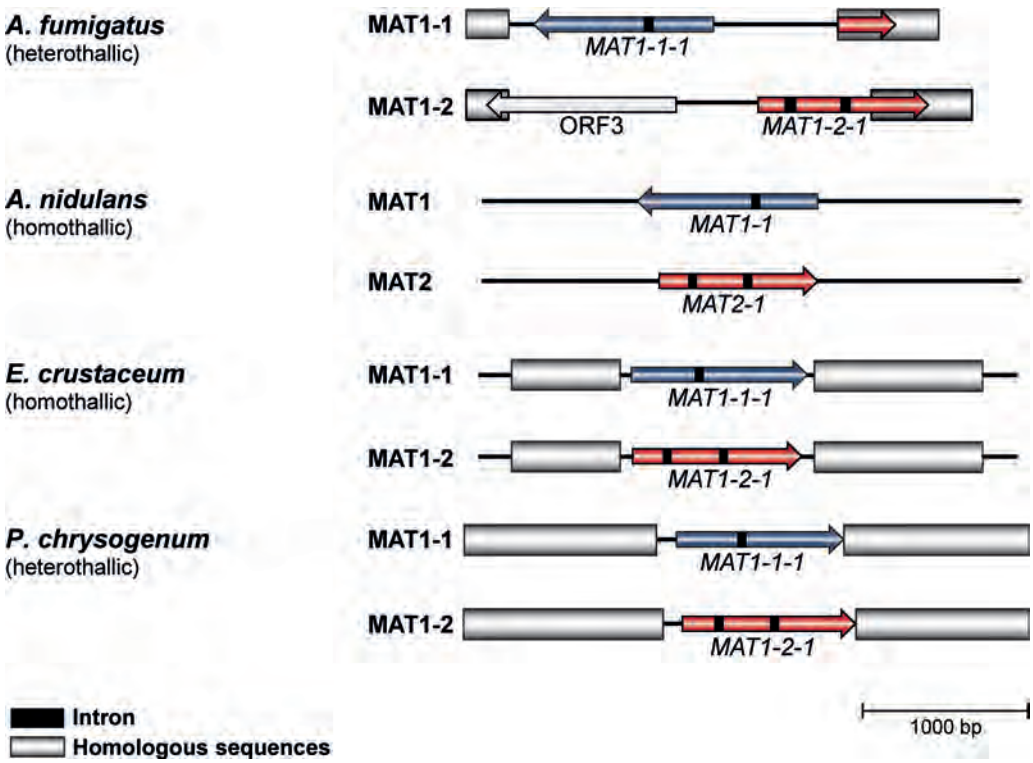


Figure 1 Comparison of *MAT* loci from homo- and heterothallic members of the Eurotiales. Blue arrows indicate a *MAT*α-domain gene, red arrows indicate a *MAT* high-mobility group gene, black bars indicate intronic sequences, gray bars homologous sequences (48, 49, 51, 173). For *A. nidulans*, the gene designation is as previously published by Paoletti et al. (49). Note that, whereas isolates of heterothallic species contain only one *MAT* idiomorph (either *MAT1-1* or *MAT1-2*), isolates of homothallic species contain both types of *MAT* gene in the same genome (i.e., both *MAT1-1* and *MAT1-2*).

phylogenetic analyses, and *in silico* predictions of secondary and tertiary structures of α and HMG transcription factors supported the hypothesis that the α1 domain is related to the HMG domain (53). This study concluded that fungal α1-box genes originated from an ancestral HMG gene.

Distribution of mating-type genes

First, mating-type genes have provided insights into the nature of asexual fungi through a population genetics approach investigating the distribution of *MAT* genes in natural populations of supposedly asexual fungi. It might be predicted that truly asexual species would be composed of near-clonal populations consisting of isolates of only one mating type as a result of speciation from a founder of a single mating type. Alternatively, genetic drift could lead to loss of a complementary mating type because there would be no selection pressure to maintain both mating types within a species. And rarely, some fungal species have been found in which isolates of only one mating type have been found (reviewed by Dyer et al. [21]). For example, all isolates of the plant pathogens *Rhynchosporium lolii* and *Rhynchosporium orthosporum* from a diverse range of geographical locations and plant hosts throughout Europe were exclusively of the *MAT1-1* genotype (54), whereas all individuals of the lichen-forming fungus *Thamnolia vermicularis* sampled from the Northern, and parts of the Southern, Hemisphere were entirely of the *MAT1-2* genotype (55). However, almost always, when studies of *MAT* distribution have been made in asexual fungal species, it has been found that isolates of both mating types can be found (i.e., the presence of both *MAT1-1* and *MAT1-2* idiomorphs), and often these are present in a near 1:1 distribution (reviewed in reference 21). This is consistent with a heterothallic sexual breeding system and has been used as strong evidence for the potential for sexual reproduction in the respective asexual species. Such studies have been made with a wide taxonomic diversity of fungi including *Aspergillus*, *Cochliobolus*, *Fusarium*, *Penicillium*, *Rhynchosporium*, and *Trichoderma* species as listed in Table 1. As an example, both *MAT1-1* and *MAT1-2* isolates of *P. chrysogenum* have been found worldwide in near equal distribution (Fig. 2). It is noted that the term heterothallic should only strictly be used for outcrossing species where a sexual state has been observed, and the term "proto-heterothallic" has been suggested to describe such asexual species where genetic evidence (e.g., the presence of complementary *MAT* loci) indicates the presence of a sexual cycle (56).

Function and regulatory impact of mating-type locus encoded transcription factors

Second, mating-type genes have provided insights into the nature of asexual fungi through an investigation of their expression and functional activity. The majority of studies have been limited to examining whether or not *MAT* genes are expressed at the mRNA level as an indication of activity. In almost all cases, *MAT* gene expression has indeed been demonstrated in imperfect fungi where assayed, which has been interpreted as providing evidence of sexual potential because the *MAT* genes would appear to be conferring sexual identity in the respective species under examination (see examples listed in Table 1). There have been some rare exceptions. For instance, it was not possible to detect *MAT1-1-1* gene expression in *R. lolii* and *R. orthosporum*, although *MAT* gene expression was detected in other supposed asexual *Rhynchosporium* species (54), but such cases are highly unusual, so far.

However, more in-depth research over recent years has revealed a rather more complex scenario, with expression analysis, providing evidence for a regulatory impact of *MAT*-encoded TFs beyond specifically sexual reproduction. Therefore, it has become clear that *MAT* gene expression alone does not necessarily indicate sexual potential, and caution should be exercised in interpreting such data, because *MAT* genes are likely to have other important functions in asexual fungi beyond mating and sexual development. This was demonstrated by pioneering *MAT*-locus-dependent expression analysis in the asexual *Aspergillusoryzae*, which showed that *MAT1-1* and *MAT1-2* specifically regulated over a thousand genes, including many of unknown function, with 33 genes differentially regulated over 10-fold between the different mating types (57). This was interpreted, in part, as evidence for *MAT* gene functional activity and therefore sexual potential, but this study also indicated that *MAT* loci are involved in the regulation of genes beyond those strictly involved in a putative sexual cycle. Similarly, gene manipulation of the *MAT1-1-1* gene from the then considered asexual *P. chrysogenum* revealed that the MAT1-1 α-domain TF impacted developmental processes of biotechnological relevance such as penicillin biosynthesis, light-dependent asexual sporulation, and hyphal morphology, in addition to any role in sex (30). This work was extended, when the *MAT1-1-1* gene from *P. chrysogenum* was analyzed by ChIP-seq analysis. This investigation led to the identification of 254 putative direct MAT1-1-1 target genes and a highly specific MAT1-1-1 DNA-binding sequence (58). Interestingly, some of the most highly bound regions in ChIP experiments

Table 1 Evidence for mating-type loci, their distribution, functional characterization, and induction of a sexual cycle in representative euascomycete species that have been presumed to be asexual[a]

Organism	Identification of *MAT* loci	1:1 distribution of *MAT* loci[b]	Functional *MAT* locus[c]	Induction of a sexual cycle[d]	Reference(s)
Acremonium chrysogenum	*MAT1-1*	No	Yes	No	77
Aspergillus flavus	*MAT1-1*, *MAT1-2*	Yes		Yes	26, 80, 158
Aspergillus fumigatus	*MAT1-1*, *MAT1-2*	Yes		Yes	28, 48
Aspergillus niger	*MAT1-1*			No	70
Aspergillus nomius	*MAT1-1*, *MAT1-2*			Yes	104
Aspergillus oryzae	*MAT1-1*, *MAT1-2*	Yes			57
Aspergillus parasiticus	*MAT1-1*, *MAT1-2*	Yes		Yes	27, 80, 159
Aspergillus sclerotiocarbonarius	*MAT1-1*, *MAT1-2*	Yes		Yes	108
Aspergillus terreus	*MAT1-1*, *MAT1-2*			Yes	106
Aspergillus tubingensis	*MAT1-1*, *MAT1-2*			Yes	107
Coccidiodes immitis, *C. posadasii*	*MAT1-1*, *MAT1-2*	Yes	Yes		160, 161
Cochliobolus victoriae	*MAT1-2*			No?	162
Diplodia pinea (*Sphaeropsis sapinea*)	*MAT1-1*, *MAT1-2*	Yes			163
Fusarium avenaceum, *F. culmorum*, *F. poae*, *F. senitectum*	*MAT1-1*, *MAT1-2*		Yes		164
Fusarium tucumaniae	*MAT1-1*, *MAT1-2*			Yes	165
Fusarium azukicola, *F. brasiliense*, *F. phaseoli*	*MAT1-1*, *MAT1-2*	Yes		No	150
Fusarium virguliforme	*MAT1-1*	No			150
Penicillium biforme, *P. camemberti* *P. nalgiovense*	*MAT1-2*				117
Penicillium chrysogenum	*MAT1-1*, *MAT1-2*	Yes	Yes	Yes	30, 51, 61
Penicillium digitatum	*MAT1-1*	No			166
Penicillium expansum	*MAT1-1*, *MAT1-2*	Yes			166
Penicillium fuscoglaucum, *P. paneum*,	*MAT1-1*				117
Penicillium pinophilum	*MAT1-1 MAT1-2*			Yes?	167
Penicillium roqueforti	*MAT1-1 MAT1-2*	Yes		Yes	31, 32
Phialocephalia fortinii – Acephala applanata	*MAT1-1 MAT1-2*	Yes			168
Talaromyces (*Penicillium*) *marneffei*	*MAT1-1*, *MAT1-2*			No	169
Rhynchosporium agropyri, *R. secalis*	*MAT1-1*, *MAT1-2*	Yes	Yes		54
Rhynchosporium lolii, *R. orthosporum*	*MAT1-1*	No	No		54
Septoria passerinii	*MAT1-1*, *MAT1-2*	Yes		Yes	170
Talaromyces amestolkiae	*MAT1-1*, *MAT1-2*	Yes		Yes	171
Trichoderma reesei (syn. *Hypocrea jecorina*)	*MAT1-1*, *MAT1-2*			Yes	29
Ulocladium spp.	*MAT1-1*, *MAT1-2*		Yes		172

[a]This list is not exhaustive but aims to provide an overview of the taxonomic diversity of species that have been examined.
[b]Where studies have been undertaken of the distribution of *MAT1-1* and *MAT1-2* idiomorphs in sample populations.
[c]As assessed by gene expression and/or functional characterization by gene manipulation.
[d]A question mark indicates some signs of sexual development, but no viable sexual spores formed.

occurred near homologs of known functional targets of the *S. cerevisiae* MATα1 protein, such as Pcppg1, a homolog of MFα1, encoding the α-pheromone, and Pcpre1, a homolog of STE3, coding for the a-factor receptor (59, 60). In addition, this analysis confirmed a large number of new MAT1-1-1 target genes, which had never been related to any MAT TF before and were assigned to functional categories beyond mating, such as asexual development, morphogenesis, amino acid metabolism, secondary metabolism, and iron metabolism (58). A summary of MAT1-1-1 target genes is depicted in Fig. 3. In parallel work, it was later shown that the MAT1-2 HMG domain TF from *P. chrysogenum* also impacted a variety of developmental processes, including light-dependent asexual sporulation, conidiospore germination, and surface properties, again in addition to any role in sex (61). A summary of MAT-controlled processes in *P. chrysogenum* is shown in Fig. 4. Comparable results were obtained with the homothallic cereal pathogen *Fusarium graminearum* which contains both *MAT1-1* and *MAT1-2* loci in the same genome. Unlike *Sordaria macrospora*, another well-studied homothallic euascomycete (62), *F. graminearum* requires all the transcripts from both *MAT* loci



Figure 2 Occurrence of both *MAT* idiomorphs in wild-type isolates from *Penicillium chrysogenum*. Blue and red dots represent strains with the *MAT1-1* or *MAT1-2* locus, respectively (C. M. O'Gorman and U. Kück, unpublished data).

for sexual development. The regulatory mechanisms controlled by *MAT* genes in *F. graminearum* were explored by several strategies, including genome-wide transcriptional profiling in various genetic backgrounds, and high-throughput gene deletions (63). Genome-wide microarray with total RNAs from *F. graminearum* mutants that lacked each *MAT* locus individually or together revealed 1,245 differentially expressed genes, including those involved in metabolism, cell wall organization, cellular response to stimuli, cell adhesion, fertilization, development, chromatin silencing, and signal transduction.

Another example of *MAT* genes regulating nonsexual processes comes from *Fusarium verticillioides*, where deletion of the *MAT1-2-1* gene led to a drastic reduction in carotenoid production in parallel with a severe decrease in photo-induced expression of genes encoding key enzymes of the carotenoid biosynthesis pathway (64). Moreover, *F. graminearum MAT1-1-1* and *MAT1-2-1* deletion mutants were shown to be reduced in virulence, although none of the *MAT* locus genes was important for plant infection, indicating that the MAT proteins may play a host-specific role in colonization of corn stalks (65). Another interesting demonstration of MAT TFs acting outside the sexual life cycle was found for the human pathogenic basidiomycete *Cryptococcus neoformans*, where the heterodimers of MAT TFs Sxi2a and Sxi1α were shown to be involved in regulation of several well-studied virulence genes (66). Based on these observations, it can be proposed that MAT TF target genes in hemiascomycetes are restricted to genes relevant for mating, whereas MAT-mediated regulation beyond sexual development might be a common feature in euascomycetes. It is conceivable that comprehensive rewiring of gene regulatory networks controlled by MAT TFs has occurred since the division of these two subclasses.

Genome Studies

Whole-genome sequencing projects have been completed for several asexual fungi over the past decade. This has allowed the genomes to be screened for the presence of genes known to be required for sexual reproduction as a way to evaluate whether the species are truly asexual. The rationale for this work is that, if a species is genuinely asexual, then it would be predicted that genes required for sexual reproduction would either accumulate deleterious mutations, or even be lost, in asexual species due to random mutation and lack of selective pressure to maintain the functional forms of the gene. Furthermore, those genes involved with the earliest stages of mating might be the most obvious candidates for mutation and/or loss to avoid unnecessary downstream metabolic investment in sex. This is

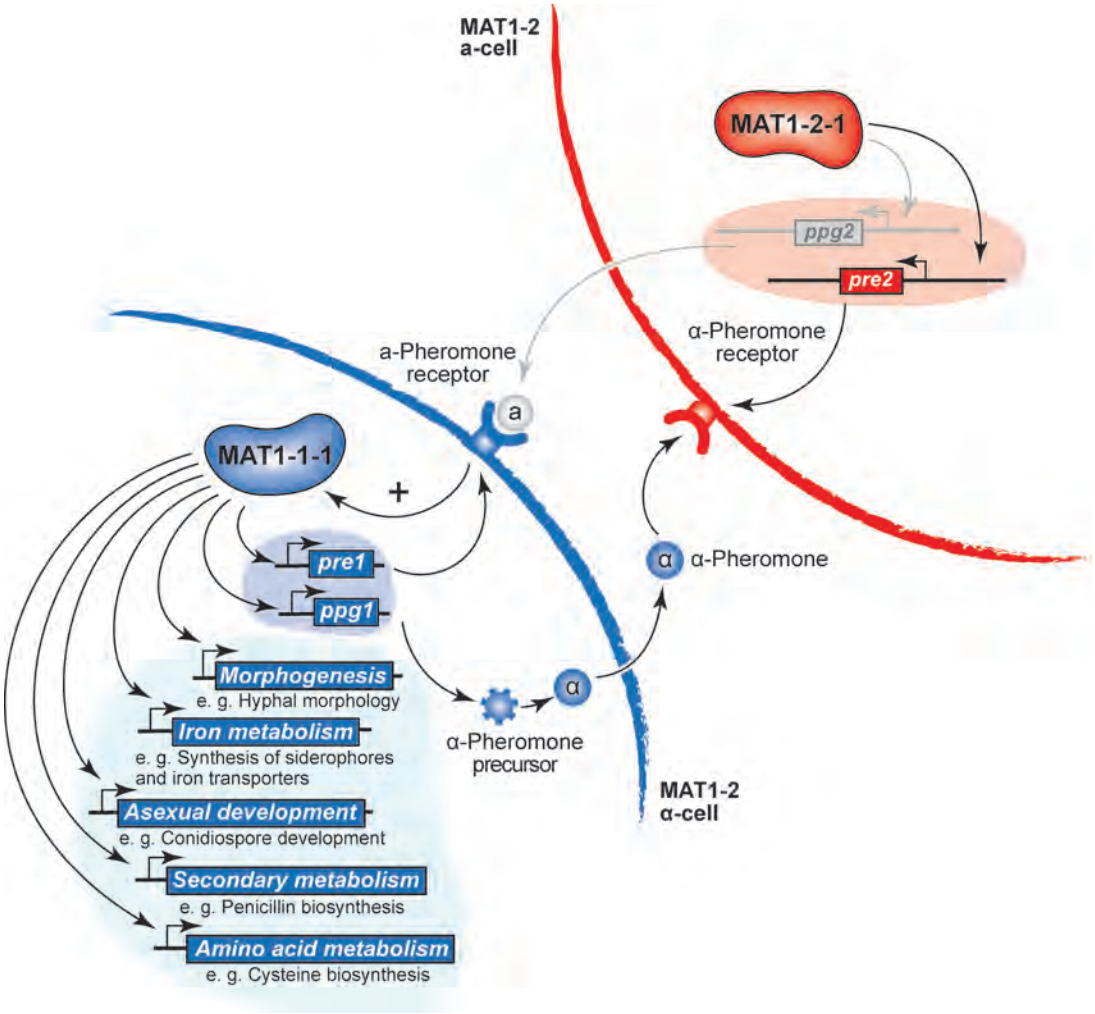


Figure 3 Target genes of the *MAT1-1-1* locus encoded transcription factors from *Penicillium chrysogenum*, deduced from functional genomics experiments (58, 61). In particular, ChIP-seq analysis has shown that MAT1-1-1 regulates gene expression far beyond their described function as regulator of sexual development (modified from reference 174).

based on the understanding that asexual species have evolved from sexual ancestors (e.g., reference 67), sex being a basal characteristic of the fungal kingdom (68).

Perhaps surprisingly, almost all genome projects to date have revealed that supposedly asexual fungi do have apparently intact sets of genes known to be required for sexual reproduction, with no evidence of gene mutation or loss. In the first study for the Pezizomycotina, the genomes of the then considered asexual *A. fumigatus* and *A. oryzae* were screened for the presence of over 200 genes linked to sex (from mating through to meiosis), and it was found that all genes were present. Furthermore, a heterothallic mating system was indicated based on the presence of a characteristic *MAT* idiomorph (69). Soon afterward, genome sequencing of the asexual *Aspergillus niger*, used widely in the biotechnological industries, again revealed the presence of a suite of genes linked to mating and sexual development (70). An apparent mutation in the *pro1* gene was found in the latter study, but this was later discounted because of an incorrect annotation of an intron. Concurrent genome screening of the then asexual *A. flavus* also revealed a full complement of apparently functional genes required for sexual reproduction (C. E. Eagle and P. S. Dyer, unpublished results). Furthermore, BLAST analysis of the genome sequence of the basidiomycete yeast *Malassezia globosa*, a causal organism of dandruff, revealed the presence of a *MAT* locus containing pheromone, pheromone receptor, and sex-linked homeodomain genes, together with

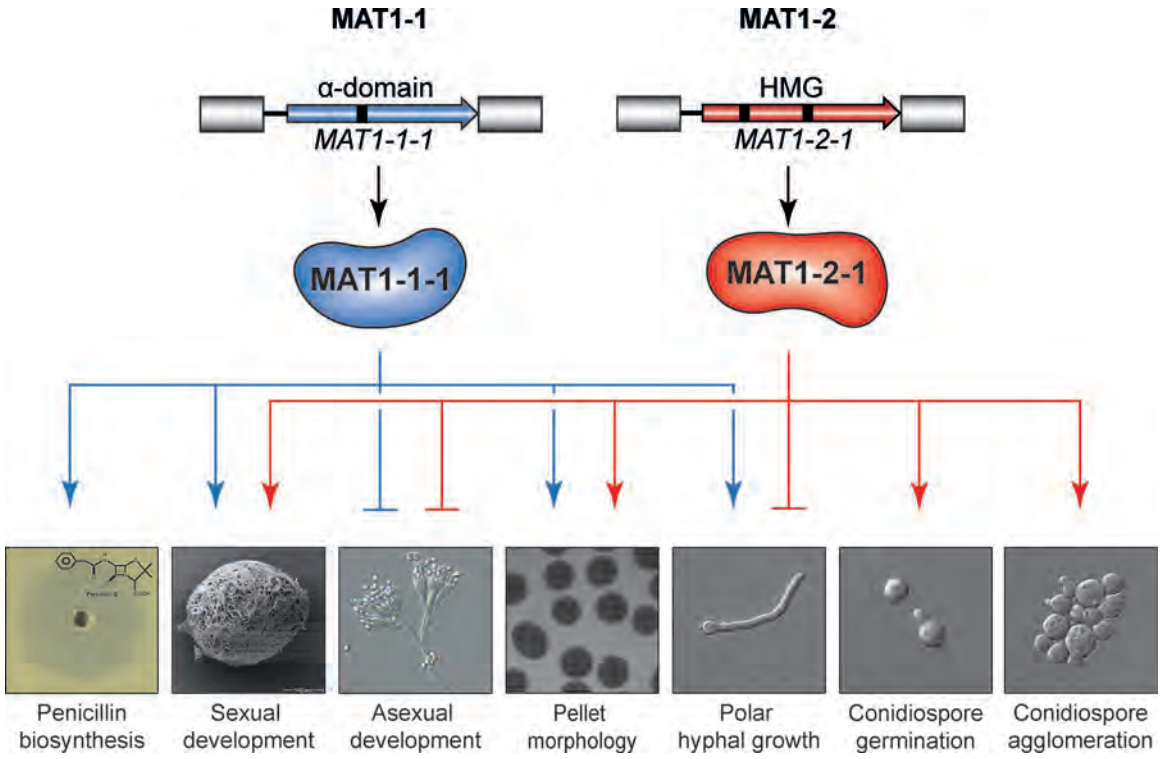


Figure 4 Summary of the regulatory functions of *MAT* locus encoded transcription factors MAT1-1-1 and MAT1-2-1 in *Penicillium chrysogenum* (modified according to reference 175).

other pheromone signaling pathway genes elsewhere in the genome of this presumed asexual species (71). Most recently, genome analysis of asexual *Rhynchosporium* species has again failed to detect any loss or mutation in genes related to sexual reproduction (54; International Rhynchosporium Genome Project, unpublished results). A further revelation from genome analysis of *P. chrysogenum* was the discovery of mutations apparently caused by a RIP-like (repeat-induced point mutation) process, again linked to sexual reproduction (72).

However, some caution must again be exercised in interpreting such evidence. Genome analysis of eight *Candida* genomes revealed that certain species with known sexual states were missing some orthodox components of mating and meiotic pathways. For example, the homothallic *Lelongisporus elongisporus* lacked any known *MAT* gene, having a syntenic *MAT* locus but which only contained noncoding DNA, and also lacked a pheromone precursor and receptor (73). Similarly, evidence of a degenerated *MAT1-1-1* pseudogene has been reported for the heterothallic *Cordyceps takaomontana* (74). Therefore, the lack of sex-related genes is not conclusive proof of asexuality, whereas the presence of such genes is arguably circumstantial evidence for sexual potential.

Functionality of Sex-Related Genes

Further evidence that imperfect fungi have the potential for sexual reproduction has come from studies where sex-related genes from certain asexual species have been expressed heterologously in model fungal species known to be sexual, and have been shown to be functionally active. This provides evidence of a conserved sexual function in the respective asexual host species. Genes assayed in this way include *MAT* genes and members of pheromone signaling pathways as will now be described.

Cross-species mating-type gene exchange provides a powerful method to demonstrate the functionality of mating-type locus-encoded proteins. Different approaches are feasible. For example, mating-type loci of the α domain or HMG type can be introduced into isolates of a heterothallic species carrying the opposite mating-type locus. In this case, the triggering of sexual development leading to formation of fruiting bodies (although not necessarily sexual spore formation) provides good evidence of *MAT* gene functionality. This

approach was successfully demonstrated in pioneering studies of known sexual species when *MAT* loci from the homothallic fungus *S. macrospora* were shown to induce sexual development (although not ascospore formation) in the heterothallic fungus *Podospora anserina* (75). An alternative approach is to use deletion mutants, lacking a mating-type locus. Complementation of the deletion mutant may be performed with a DNA fragment carrying the *MAT* locus from a heterologous host. For example, *MAT1-2-1* from *A. fumigatus*, encoding a HMG box mating transcriptional factor, was introduced into a mating-type deletion mutant of the homothallic fungus *A. nidulans* (76). Using a *MAT1-2-1* gene under the expressional control of the *Aspergillus nidulans MAT2* (*matA*) promoter, it was demonstrated that the *MAT1-2-1* gene from *A. fumigatus*, at that point only known to be asexual, was able to drive sexual reproduction in *A. nidulans*. In parallel studies, it was shown that the *MAT1-1-1* gene from *A. fumigatus* was able to complement an *A. nidulans MAT1* (*matB*) deletion mutant and restore sexual fertility (50). Comparable results were reported for *Acremonium chrysogenum*, the fungal producer of the pharmaceutically relevant β-lactam antibiotic cephalosporin C. This filamentous fungus is classified as asexual, because no direct observation of mating or meiosis has yet been reported. The investigated strains were shown to carry a *MAT1-1* mating-type locus with a *MAT1-1-1* α-domain gene and a *MAT1-1-2* gene, encoding an HPG domain protein, defined by the presence of the three invariant amino acids histidine, proline, and glycine. In addition, a *MAT1-1-3* gene was detected, encoding a high-mobility group (HMG) domain protein (77). So far, only a single wild-type isolate has been characterized, and no reports are available that strains with opposite mating-type loci exist. To assess the potential of *A. chrysogenum* for sexual reproduction, the entire *A. chrysogenum MAT* locus was transferred into a *MAT1-1* deletion strain of the heterothallic ascomycete *P. anserina*. After fertilization with a *P. anserina MAT1-2* (*MAT2*) strain, the corresponding transformants developed fruiting bodies with mature ascospores. These results demonstrated that the *MAT* genes of *A. chrysogenum* were functional and supported the hypothesis of an extant sexual cycle in this species.

Other evidence for a sexual cycle stems from functional studies of pheromone and pheromone receptor genes, using the heterologous *S. cerevisiae* system as a genetic tool. Successful pheromone binding and signaling would be expected to result in cell cycle arrest and change in cell morphology and lead to formation of a halo in lawns of *S. cerevisiae* (78). Based on similarity to the *S. cerevisiae* MFα proteins, the *P. chrysogenum* Pcppg1 gene was predicted to produce a decapeptide pheromone of sequence KWCGHIGQGC, expected to bind to the cognate PcPRE2 receptor protein. And indeed, *S. cerevisiae* wild-type cells (ScSTE2p) or yeasts heterologously expressing PcPRE2 exhibited polarized growth, leading to pear-shaped forms (shmoos, originally named after a cartoon character of this shape drawn by Al Capp) of unconjugated haploid cells, in response to either the native *S. cerevisiae* α factors or the synthetic decapeptide pheromone PcPPG1, respectively (30). This result was complemented by bioassays in which adding synthetic PcPPG1 pheromone to lawns of yeast cell, expressing PcPRE2, resulted in halo formation. Thus, PcPRE2 and PcPPG1 represent a functional pheromone-receptor pair likely involved in the mating of *P. chrysogenum*, which was later observed by Böhm et al. (30). These data were consistent with microarray analyses demonstrating the expression of the genes for a pheromone precursor (Pcppg1) and two pheromone receptors (Pcpre1, Pcpre2) in *P. chrysogenum* (30).

As well as the examples above, a STE12 homolog from the asexual *Penicillium marneffei* (renamed *Talaromyces marneffei*) was able to complement a *steA* gene deletion mutation of *A. nidulans* and restore sexual fertility (79). Meanwhile, overexpression of the *A. fumigatus nsdD* gene led to increased development of sexual structures (cleistothecia) in *A. nidulans* (50). Finally, before the identification of any sexual stage, pheromone precursor and receptor genes were shown to be expressed in *A. fumigatus*, *A. flavus*, *A. parasiticus*, *A. oryzae*, and *P. chrysogenum* in what was termed "sexual posturing" (22, 48, 51, 57, 80). Most recently, similar expression of pheromone signaling genes has been demonstrated in several other asexual aspergilli including *Aspergillus aculeatus*, *A. brasiliensis*, *A. clavatus, A. niger, A. sydowii, A. wentii*, and *A. zonatus* (178). However, as above, some caution must be used when interpreting such results because it appears that some aspects of the cellular sexual machinery can be subverted for other purposes in fungi. For example, the STE2 pheromone receptor of the asexual *Fusarium oxysporum* has been shown to play a role in detection of plant exudates (81). Therefore, detecting expression of sex-related genes is not necessarily a sign of cryptic sexuality.

Formation of Sex-Related Structures and Lichen Species Pairs

One final line of evidence that imperfect fungi have the potential for sexual reproduction comes from the observation that certain asexual species have been

observed to form developmental structures representing the initial stages of sexual reproduction. This suggests that they might have the potential for sexual fertility if correct conditions can be identified to induce the complete sexual cycle (56). For example, several asexual *Aspergillus* and *Penicillium* species are known to form sclerotia, a necessary prerequisite for sexual reproduction in closely related species (82, 83). This phenomenon was recently observed for certain isolates of *A. niger* which were able to form sclerotia when they were incubated on certain organic substrates including raisins (Fig. 5) (84) (G. Ashton and P. S. Dyer, unpublished results).

A particularly intriguing phenomenon related to this is the occurrence of so-called "species pairs" in lichen biology. This refers to the situation where two morphologically and phylogenetically very closely related pairs of lichen species have been described which differ solely in the fact that one species bears sexual structures on the thallus, whereas the sister species lacks any sexual structures (85–87). It is tempting to speculate that the reason for asexuality in the sterile partner species might be principally because of the lack of a suitable mating partner to induce sex, rather than these being genuinely separate species as per received orthodoxy. In related examples from lichen-forming fungi, Seymour et al. (86) reported finding the first fertile example of *Usnea sphacelata* (as evidenced by formation of sexual fruiting bodies [ascomata] on the thalli), and Greenaway (88) described the first fertile thalli of *Normandina pulchella*; both species had previously been considered to reproduce only by asexual means.

DISCOVERY OF MATING AND SEXUAL REPRODUCTION—AND IMPLICATIONS

The above lines of evidence have provided, and these continue to give, an indication of the potential for sexual reproduction in the imperfect fungi, although such evidence has at best been circumstantial because the elusive sexual states were still missing. However, a series of major breakthroughs have been reported since 2000 for yeasts and 2009 for filamentous fungi, where modern molecular approaches combined with classical microbiology laboratory culture have been used to demonstrate the existence of either mating and/or a complete functional sexual reproductive cycle in a series of supposed asexual species from different genera. In most cases, a key first step was the use of molecular diagnostic tools to identify potential sexual mating partners and then the setting up of directed crosses between parental isolates of complementary mating type under a range of incubation conditions (56). Some notable examples are described below.

Mating in *C. albicans*

There had been strong indications that the opportunistic human pathogen *C. albicans* might have sexual potential following the identification of a mating-type locus similar to that seen in the model sexual yeast

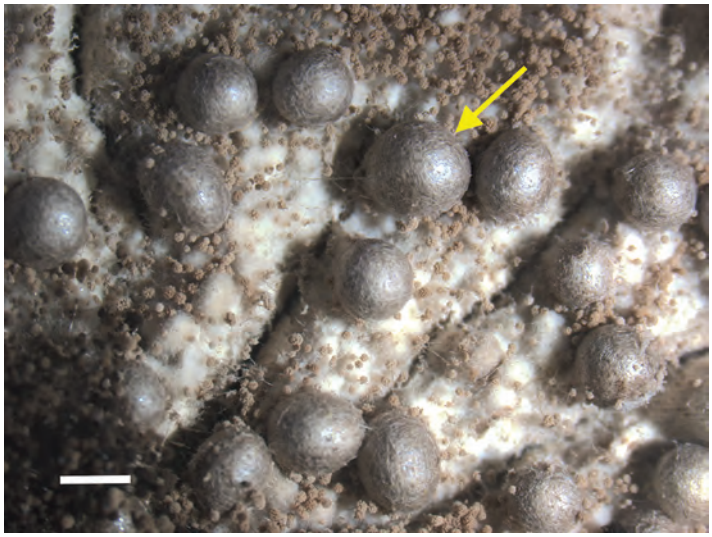


Figure 5 Sclerotia formation (arrowed large gray-brown spheres) in *Aspergillus niger*, an indication of the potential for sex in this biotechnologically important species? Scale bar indicates 500 μm. Note that this species is predominantly of the *MAT1-1-1* genotype (H. Darbyshir, G. Ashton, and P. S. Dyer, unpublished data).

S. cerevisiae, together with the detection of other genes that had high homology to sexual pathway genes in *S. cerevisiae* (89) (see also Bennett and Turgeon [177]). These indications were confirmed when two different groups were able to induce mating between **a** or α strains of *C. albicans* to produce a tetraploid state. Hull et al. (24) made artificial **a** or α strains by deletion of the alternative MTL α or **a** locus, respectively, and crossed these under selective conditions in mice, while Magee and Magee (25) induced chromosome loss to produce **a** or α strains by deletion of one of the MTL loci and then crossed auxotrophic strains on agar media using selective conditions. Although both groups therefore reported the first success in mating strains of *C. albicans*, the rates of mating were rather low and led to a tetraploid state without any meiotic division. A second major advance then followed, being the discovery that homozygous **a/a** or α/α *C. albicans* cells must undergo a phenotypic transition from a "white" traditional yeast-like morphology to an "opaque" morphology to achieve mating competency (90–92). However, even under these conditions, meiosis has still not yet been observed in *C. albicans*. Instead, tetraploids appear to undergo stochastic concerted chromosome loss to return to a diploid or near-diploid state, particularly under stressful conditions (93, 94). Furthermore, even when mating-competent haploid strains of *C. albicans* were produced under *in vitro* and *in vivo* conditions, it was still not possible to induce meiosis (95). Therefore, true sex involving meiotic nuclear recombination has still not yet been observed in *C. albicans*; instead, this is best described as mating and a parasexual process. It is also noted that the intriguing phenomenon of same-sex mating has also been observed in *C. albicans*, but again with no evidence of meiosis (96).

Sex in *Aspergillus* Species

The genus *Aspergillus* comprises over 340 species (97) and includes a series of species of great economic, medical, and scientific importance. Members of the genus share the common feature of an "aspergillum," a branching head conidiogenous structure for the production of asexual spores. In addition, about one-third of species (36% according to reference 33) are known to reproduce sexually. All the latter species produce fruiting bodies known as cleistothecia, but a variety of cleistothecial forms are evident, meaning that under traditional taxonomic classification, different teleomorphic genera have been assigned according to morphological features such as whether cleistothecia are enclosed, the color of cleistothecia, and whether they are surrounded by accessory cells (33, 37, 97). The fact that the remaining majority of *Aspergillus* species (64% according to reference 33) are only known as imperfect fungi has attracted research interest both from an applied and fundamental perspective, because many species of biotechnological and pathological interest were only known as asexual organisms. If it were possible to induce sexual cycles in such species, then this would provide a valuable tool for strain improvement and classical genetic analysis, as well as providing insights into the population biology and evolutionary potential of species. Research into this area has been reviewed (see reference 37), with a summary and updates provided below.

Initial investigations involved the use of population genetic approaches, which revealed evidence of high levels of genetic diversity and recombination in nature for the spoilage and aflatoxin producing *A. flavus* and *A. parasiticus* (98, 99), and the opportunistic pathogen *A. fumigatus* (28, 48, 100, 101). Mating-type genes were then identified for the first time from the aspergilli (102, 103), which allowed PCR diagnostics to be developed. This led to the identification of alternative *MAT1-1* and *MAT1-2* idiomorphs and the discovery of a near 1:1 distribution of compatible mating types both within global and regional populations of *A. fumigatus* (28, 48). Similar results were later reported for both *A. flavus* and *A. parasiticus* for a local field population from the United States (80). All these observations provided evidence of cryptic sexuality in these species.

A major breakthrough then came when laboratory work led to a sexual stage being induced for the first time in *A. fumigatus*. This was achieved by crossing known *MAT1-1* and *MAT1-2* isolates from a population in Ireland (which had shown evidence of recombination) on oatmeal agar in a barrage formation under specific environmental conditions. Cleistothecia characteristic of the teleomorph genus *Neosartorya* were produced after 6 to 12 months, and the sexual stage named *Neosartorya fumigata* under existing taxonomic rules. Recombination was demonstrated in the ascospore offspring using molecular markers (28). Soon afterward, similar crossing efforts were rewarded with *A. flavus* and *A. parasiticus*, when incubation of strains of opposite mating type for between 6 and 12 months led to the production of hardened sclerotia found to contain sexual ascospores, although recombination was not assayed. The two sexual stages were assigned to the teleomorph genus *Petromyces* (26, 27).

The studies with *A. fumigatus, A. flavus*, and *A. parasiticus* provided a precedent for ensuing research in which attempts were made to cross *MAT1-1* and *MAT1-2* isolates of other asexual *Aspergillus* species

under conditions conducive to sex in the aspergilli. A sexual cycle was subsequently induced in *A. nomius* (104), *A. lentulus* (105), *A. terreus* (106; A. E. Ahmed and P. S. Dyer, unpublished results), *A. tubingensis* (107), *A. sclerotiicarbonarius* (108), and *A. clavatus* (S. Swilaiman and P. S. Dyer, unpublished results). The discovery of sexual reproduction in *A. tubingensis* and *A. sclerotiicarbonarius* is particularly notable, because these are members of the black aspergilli and therefore related to the economically important species *A. niger*, which is much used in the biotechnology sectors. However, all efforts to induce sexual reproduction have so far failed in this species, although the ability to induce sclerotial production in certain strains, together with identification of a TF linked to this process, is a promising sign (Fig. 5) (84; T. R. Jørgensen, J. Frisvad, K. F. Nielsen, P. S. Dyer, and A. F. J. Ram, unpublished results).

Sex in *Penicillium* Species

The genus *Penicillium* comprises over 360 species that have a ubiquitous distribution (83). Morphologically, they are characterized by the production of an asexual reproductive structure, called the penicillius (83, 109). Together with species of the genus *Aspergillus*, penicillia belong to the order *Eurotiales* (97) and are among the most prevalent fungi on earth. Taxonomically, *Penicillium* species are the asexual (anamorphic) forms of sexual (teleomorphic) species from the genera *Eupenicillium* and *Talaromyces* (83, 97). However, the majority of *Penicillium* species (73% according to reference 33) have no known sexual state.

Because of their widespread beneficial usage in food production and biotechnology, penicillia are of outstanding economic and medical importance. For example, *P. camemberti* and *P. roqueforti* are used in the making of white mold (e.g., Brie and Camembert) and blue mold-ripened cheeses (e.g., Roquefort and Stilton) (110). In the pharmaceutical industry, *P. brevicompactum* is used for large-scale production of the immunosuppressant mycophenolic acid, *P. griseofulvum* for the industrial production of the broad-spectrum antibiotic griseofulvin, and *P. citrinum* for production of the cholesterol-lowering drug compactin (111–113). Last but not least, *P. chrysogenum*, perhaps the most renowned representative of the genus *Penicillium*, constitutes the only industrial producer of the β-lactam antibiotic penicillin. Today, penicillin is the most commonly used drug in the treatment of bacterial infections and one of the most valued products in the global anti-infective market. As with the aspergilli, the economic importance of certain asexual *Penicillium* species has meant that they have attracted research interest regarding the possibility of inducing sexual reproduction for strain improvement and classical genetic applications as will now be described for *P. chrysogenum* and *P. roqueforti*.

Initial investigations of the sexual potential of *P. chrysogenum* (reclassified recently as *P. rubens* by Houbraken et al. [97, 114]) were made using DNA and genome-sequencing approaches (51, 115). Interestingly, the strain originally discovered by Sir Alexander Fleming (116) was found to contain a *MAT1-2* locus, while later, industrially used strains were found to carry the *MAT1-1* locus. The latter observation can be explained by the fact that they all are derivatives of a wild-type isolate, obtained in the late 1940s in Peoria, Illinois. Hoff and coworkers showed that these strains retained transcriptionally expressed pheromone and pheromone receptor genes required for sexual reproduction (51). A further survey showed that *P. chrysogenum* isolates from diverse global locations contain either the *MAT1-1* or the *MAT1-2* locus with an almost equal distribution (Fig. 1).

In order to induce the sexual life cycle in *P. chrysogenum*, 17 *MAT1-1* wild-type or mutant strains were crossed in various combinations with 10 *MAT1-2* wild-type isolates using a range of different growth media and conditions. Finally, a *MAT1-1* strain derived from a strain development program in the 1950s was found to generate cleistothecia in crosses with a *MAT1-2* wild-type strain (30). The culture conditions were similar to those described for *A. fumigatus* (28), but the oatmeal agar had to be supplemented with biotin, which accelerated the formation of mature cleistothecia, which appeared after 5 weeks. Measurement of the penicillin titer on plate test and examination of restriction fragment length polymorphism of 11 genes, which differed between the two parental strains, proved that genetic recombination has occurred in all ascospore isolates investigated. In a subsequent analysis, the chromosome constitution of selected ascospore isolates was determined by whole genome sequencing (61). Included in these studies were the functional analyses of deletion strains, lacking either the *MAT1-1* or the *MAT1-2* locus. From these studies, it became evident that both mating-type loci have functions beyond sexual development (see discussion above).

Meanwhile the sexual potential of *P. roqueforti* has also been investigated. *P. roqueforti* is widespread in the natural environment and is a common spoilage agent in stored foods and meat products. In food production, this ascomycete is used as a starter culture for blue-veined cheese production. A survey of several

world isolates from *P. roqueforti* revealed the presence of both mating types, and the detection of repeat-induced point mutation (RIP) within repeated sequences and transposable elements indicated strongly that sexual recombination had occurred or was ongoing in *P. roqueforti* (31, 117). This indirect evidence for a sexual cycle was recently confirmed when a sexual cycle was induced under laboratory conditions (31, 117; Swilaiman et al., unpublished). In one study, four *MAT-1-1* and *MAT1-2* strains were tested in eight possible mating combinations. Although many combinations were not fully fertile (only empty fruiting bodies were produced), typical sexual structures such as cleistothecia, asci, and ascospores were detected in some crosses (32). In a parallel study, sexual reproduction was induced in a higher number of crosses, including when crossing isolates from different types of blue cheese (31; Swilaiman et al., unpublished). Interestingly, the conditions developed for *P. chrysogenum* (30) to induce sex were identical, indicating that these conditions are valid for a wide range of *Penicillium* species. Recombination of ascospore progeny from *P. roqueforti* was demonstrated with either microsatellite or DNA fingerprint markers (31, 32; Swilaiman et al., unpublished). The sexual cycle of *P. roqueforti* offers the exciting potential to select for progeny with altered flavor and mycotoxin characteristics for novel blue cheese strains (H. Darbyshir, J. Frisvad, and P. S. Dyer, unpublished results).

Sex in *Trichoderma* and Other Genera

The ascomycete *T. reesei* is used in the biotechnology industry for applications such as the decomposition of diverse substrates through to the production of second-generation biofuels from cellulosic waste. Moreover, efficient biocontrol strains have been developed as biological fungicides (118). *T. reesei*, the anamorph of the ascomycete *Hypocrea jecorina*, was believed for more than 50 years to propagate strictly asexually. However, Seibel and coworkers succeeded in inducing sexual development in the industrial production strain QM6a (29). This strain contains the *MAT1-2* mating-type locus, but was found to be female sterile, i.e., unable to produce the specialized hyphal tissues needed for fruit body development (in this case, perithecia). Thus, in mating, this strain was only able to act as a male fertilizing partner, but was then able to undergo a heterothallic reproductive cycle with wild-type isolates carrying the *MAT1-1* locus. Perithecia embedded in stroma were generated under different light conditions. Interestingly, visible light is dispensable for stroma formation, and constant light even inhibited stroma formation. Further studies indicated that the light-regulatory protein Envoy 1, a homolog of the light-oxygen-voltage (LOV) domain-containing protein vivid from *N. crassa*, is essential for female fertility (119). In follow-up studies, further parental strains were crossed with *T. reesei*. The analysis of unusual 16-spore asci, which were produced when parental strains showed chromosomal heterogeneities, revealed the formation of a considerable number of inviable ascospores. This indicated that chromosomal homogeneity is crucial for highest sexual fertility (120). These results correspond well with the sequencing data of ascospore progeny of *P. chrysogenum* that were derived after crossing of parental strains that exhibited heterogeneous chromosomal configurations (121).

There are further reports of sex being induced in other genera such as *Fusarium* (122) and *Trichophyton* (123) species by utilizing *MAT* diagnostic tools and particular crossing conditions, but a full discussion is beyond the scope of the present review.

Implications of the Discovery of Fungal Sex

The presence of a sexual cycle offers many potential benefits. First, the discovery of a sexual cycle in biotechnologically relevant species provides an exciting new tool in addition to conventional breeding approaches to genetically engineer industrial production strains in the laboratory. Thus, fungal mating systems may be appropriate for the improvement of industrial production strains similar to established animal and plant-breeding programs (124). This strategy was recently implemented for *T. reesei*, when a female sterile strain was engineered to female fertility (125). By introduction of the *ham5* gene, it was possible to restore female fertility of the QM6a *T. reesei* wild-type strain, which is the progenitor strain of all industrially used derivatives. The generation of this strain, which may serve as male as well as female partner in mating experiments, provides the basis for further breeding of this cellulose-producing fungus. As an indication of the potential for this work, a crossing experiment with different wild-type mating partners of *T. reesei* showed that ascospore isolates were obtained that produced a higher level of xylanases than either parental strain, despite chromosomal heterogeneity (120). In comparable work, low-penicillin-producing strains could be crossed with *P. chrysogenum* wild-type isolates to give rise to ascospore isolates with an increased penicillin titer (61). By crossing different isolates of *P. chrysogenum* it was also possible to select for ascospore progeny with minimal production of a secondary metabolite chrysogenin, which can interfere with penicillin purification (30).

However, it might prove to be a general problem in future mating experiments to obtain mating partners with compatible karyotypes. Previous crossing experiments with isolates from different locations of the pseudohomothallic ascomycete *Neurospora tetrasperma* resulted frequently in sexual dysfunction, with abnormalities ranging from reduced ascospore viability to complete sterility (126). Pulsed-field gel electrophoresis analysis and mating of different geographic strains from the homothallic fungus *S. macrospora* showed a correlation between karyotype heterogeneity and reduced sexual fertility (127). Similarly, one investigation of mating with *P. roqueforti* strains from noncheese and cheese habitats belonging to different genetic clusters found that highest fertility was only achieved when strains from the same genetic cluster were crossed. Fertility was significantly reduced when strains isolated from cheese were crossed with those from noncheese habitats, possibly as a result of domestication and clonal selection (128). However, parallel studies found comparable levels of fertility when crossing different isolates of *P. roqueforti* used in blue cheese production, so this might be a strain-specific effect (31; Swilaiman et al., unpublished). In future strain improvement programs, the combination of conventional mating experiments and contemporary molecular tools, such as ChIP-Seq or forward genetic analysis, will generate novel approaches to engineer genetically industrial producer strains. One prerequisite for the success of these breeding experiments might therefore be to select or design mating partners with compatible karyotypes to ensure breeding success.

Second, the discovery of a sexual cycle provides a very valuable tool for classical genetic analysis. By crossing of partners showing different traits and analysis of the sexual progeny, it is possible to determine whether a trait is mono- or polygenic in basis. The sexual cycle can also be used for gene mapping, verification of gene function, and identification of genes of interest through techniques such as bulk segregant analysis (124).

Finally, the discovery of a sexual cycle provides important insights into the evolutionary potential of species. Species undergoing sexual recombination are likely to generate and maintain considerable genetic diversity within populations. This is predicted to lead to more rapid breakdown of control measures for fungal pathogens (129). Therefore, having knowledge about the reproductive mode of a species can inform disease management strategies. This might, for example, involve removal of suitable substrates to reduce the risk of occurrence of the sexual cycle—as can be achieved by removal of straw stubble after harvest to prevent the sexual cycle of plant pathogenic *Tapesia* species (130), and melon debris infected with *Monosporascus cannoballus* after crop termination (131).

POTENTIAL FOR SEX IN OTHER ASEXUAL SPECIES—CONSIDERATIONS

As will be apparent from the examples given above, in almost all cases where asexual fungal species have been studied in depth, evidence can be obtained of cryptic sexuality, and, in many instances, a fully functional sexual cycle can be induced. This raises the question of whether there are any truly imperfect fungi. We offer some considerations about this topic below.

First, we contend that there are some genuinely long-lived asexual fungal species, which is made possible by the genetic flexibility that is an especial characteristic of the fungal kingdom (132). Fungi exist in nature in a variety of ploidy states, from haploid to diploid to tetraploid, they can exhibit accessory chromosomes, and some species can even manipulate the genome to duplicate localized chromosome regions in the face of environmental pressures (132, 133). In tandem with this, fungi have developed alternative methods to heterothallic sex to allow gene flow both within and between species. Fungi are able to undergo vegetative fusion with hyphae of partners of the same heterokaryon compatibility group, thereby allowing nuclear exchange, and this can even lead to nuclear fusion and gene recombination via the parasexual cycle (see Daskalov et al. [176]). In recent years there have been reports of same-sex mating in human pathogenic fungi (92, 96). Also, there is accumulating evidence of horizontal gene transfer in the fungal kingdom both from fungi and also prokaryotic organisms (134). In early reports, transfer of low-molecular extrachromosomal DNA was reported between *Neurospora* species, between the distantly related ascomycetes *Ascobolus immersus* and *P. anserina*, and from the zygomycete mycoparasite *Parasitella parasitica* to its host *Absidia glauca* (135–137). Therefore, there is arguably less pressure on fungi to be sexually "perfect" to provide new sources of genetic variation than in many other eukaryotic organisms where there is an obligate requirement for sex.

What characteristics might such long-lived imperfect fungal species have? We would suggest factors such as the lack of any observed sexual cycle or developmental structures despite sustained investigative studies; a high degree of clonality in populations; loss of genes normally associated with sexual development or their

subversion to other cellular functions; dominance of one mating type (if *MAT* loci could even be detected); and development of alternative methods for generating or sustaining genetic variation as an adaptation to asexuality. The predicted long-term genomic effects of the loss of sex have even been classified and include possible congruent divergence of nuclear loci (the "Meselson effect" in asexual diploids), decrease in mutation rate, and loss of transposable element activity (see Table 1 in Normak et al. [4]). As a prime example in the fungal kingdom, we propose the group of fungi involved with vesicular-arbuscular mycorrhizal (VAM) symbioses from the phylum Glomeromycota (138). No morphological evidence of sexual structures has ever been observed in this group, which, combined with clonal population structures and conservation of asexual spore morphology, has led to the suggestion that they have been asexual for over 400 million years, even though some core meiotic genes have been detected (7, 139–141). Instead, it appears that individuals can arise from heterokaryotic spores composed of a mosaic of nuclei, which can maintain and promote genetic variation that helps explain occasional signs of recombination seen in the VAM fungi (142, 143). Such nuclear variation might provide a useful way to buffer these apparently asexual organisms against the effects of accumulating mutations (144) and maintain genetic diversity in the absence of sex. Most recently, a putative mating-type locus has been identified from genome sequencing of the model species *Rhizophagus irregularis*, suggesting a sexual origin for the observed heterokaryosis (145).

Second, we suggest that certain human activities have resulted in an increased likelihood of the evolution of asexual fungal species. Despite the many benefits of sexual reproduction, there are also costs to sexual reproduction leading to fungal "sexual hangups" (15). In outcrossing sex for every good, enhanced individual in the sexual offspring, there will be a corresponding poorly adapted one with less favorable sets of genes compared with the parents. Indeed, there is some evidence from comparing offspring of outcrossing and selfing in *A. nidulans* that outcrossing can result in progeny with an overall mean reduction in fitness, albeit that some offspring have significantly increased fitness (146). Thus, sex involving outcrossing has the risk of breaking up favorable sets of genes, a factor that can provide strong selection pressure for either the evolution of asexuality and/or homothallism in organisms well adapted to their environment (147). Human activities can exacerbate this phenomenon by establishing large, relatively uniform environments in which well-adapted organisms might evolve away from sex to avoid the risk of dilution of favorable genomes. There are also increased metabolic costs to sexual reproduction compared with asexual reproduction as a result of the investment needed for mating and development of fruiting bodies. In addition, the number of sexual spores produced is normally far less than can be produced by an equivalent investment in asexual reproduction, and development of such sexual spores needs a longer time period because of the complexity of sexual morphogenesis, all further reasons for the evolution of asexuality. Thus, there can be benefits to an asexual lifestyle. As Ross (148) wryly commented, the thought that the absence of sex is an imperfection, and that sex is perfection, might be an assumption of the human observer, rather than the fungus!

An example of such human activity perhaps favoring the evolution of asexuality is the deployment over wide geographic regions of crop plants with a narrow genetic base that has provided the opportunity for the clonal spread of fungal plant pathogens, which if well adapted to their host have an incentive to lose sex for all the factors listed above. And, indeed, there is evidence of what has been termed a "slow decline" in sexual fertility in populations of some fungal phytopathogens (149). *F. oxysporum* has evolved to be pathogenic on a wide variety of plant hosts and exhibits clonal spread on some crops such as banana. Despite concerted efforts, it has not been possible to induce a sexual cycle in this species complex, and pheromone-signaling sex-related genes now appear to be involved with pathogenicity processes detecting a plant host (81). A similar evolution away from sex is apparent for several other *Fusarium* species (122, 150). Similarly, different *Rhynchosporium* species (causing leaf blotch disease) are thought to have evolved on various graminaceous hosts as a result of human agricultural activities linked to crop selection, cultivation, and then dispersal over recent millennia (151, 152). These host-specialized species are only known to reproduce asexually, and sampling of *R. lolii* and *R. orthosporum* has so far only detected isolates of a single mating type (54). Finally, the rice pathogen *Magnaoporthe oryzae* is considered to be a heterothallic sexual species. However, there are reports of extensive loss of sexual potential in field populations worldwide, both due to loss of female fertility (i.e., the ability to produce maternal fruit body tissues, while rudimentary male ability to donate nuclei is retained) or total loss of any mating ability (153, 154). The slow decline in sexual ability might be attributable to various factors, as previously discussed (149), and the loss of sexual fertility seen in the field mirrors that seen

under laboratory conditions when isolates are subcultured long-term by asexual means (149, 155). The long-term future of such asexual species is unclear; it may be speculated that they are still likely to be relatively short-lived evolutionary dead ends (18) because they are dependent on human activity.

Third, we suggest that although some asexual species do exist and others are currently evolving, overall such species are still likely to be very rare. Therefore there is likely to be the occurrence of a sexual cycle in the majority of 20% of all fungi that are currently only known in the imperfect state. We suggest that there are various reasons for the failure to detect sex in such species. Many might need very specific conditions to induce a sexual cycle, requiring much painstaking work to identify (56). A further obstacle is that almost all are likely to exhibit heterothallic breeding systems, requiring the identification of a possibly rare compatible mating type as is the case for *C. neoformans* and *Cryptococcus gattii* (92), whereas it will be much easier to induce sex in homothallic species where no mating partner is needed. For some species, many isolates might have low sexual fertility as a result of a slow decline in sexual potential in the species overall (149). Therefore, it might take dedicated sampling to find rare isolates of a species that are a remnant that maintain sexual potential. But such isolates would be important in evolutionary terms to generate variation episodically within populations. Indeed, given the argument that "a little sex goes a long way," (7) it may only be necessary to retain a low proportion of isolates that retain the ability to reproduce sexually, i.e., that a very low rate of sexual recombination is sufficient to overcome evolutionary problems associated with asexuality.

CONCLUSIONS

With the advent of the "one species, one name" ruling in fungal biology (156), whether a species is perfect or imperfect has less taxonomic relevance than the past, because only one Latin binomial is needed for a species regardless of sexuality. But a knowledge of the reproductive mode remains a critical feature for an understanding of the biology of a species. There has been a sexual revolution in the understanding of asexuality over recent years, with a number of studies revealing sexual cycles in species that were previously considered imperfect. This implies that the majority of asexual fungal species will be shown to exhibit cryptic sexuality with the potential for sexual reproduction. However, there is a danger of simply thinking of fungi as either "perfect" sexual or "imperfect" asexual species. Indeed, we finish by echoing the comments of Dyer and Paoletti (149) that species should not be viewed as either sexual OR asexual, but, rather, they should be thought of as consisting of isolates on a continuum of sexual fertility, with the potential presence of isolates within a species with a range of abilities to undergo sexual reproduction from high to low fertility to even apparent asexuality. A thought to ponder over a cup of coffee, appropriate given that caffeine can induce sex in some fungal species (157).

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Molecular Mechanisms Regulating Cell Fusion and Heterokaryon Formation in Filamentous Fungi

10

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INTRODUCTION

Cell-cell fusion is an essential biological process that occurs in organisms throughout the tree of life. It is involved in both sexual and asexual developmental processes in most species and has been shown to occur in multicellular and in unicellular organisms. Somatic cell fusion events are widespread in eukaryotic organisms, including animals, where they are important for muscle differentiation, placental development, and formation of multinucleate giant cells in the immune system (1–4).

Somatic cell fusion is a highly regulated event that requires reciprocal recognition as well as coordinated molecular processes in fusion partners. However, indiscriminate cell fusion comes with risks, including fusion with infected, impaired, or genetically different fusion partners, which can result in a disadvantaged heterokaryon. Thus, cells have evolved mechanisms to discriminate genetically identical from genetically nonidentical (nonself) cells. There are diverse types of genetic recognition mechanisms that organisms use to distinguish self from nonself. In mammalian cells, self/nonself recognition mechanisms mediate pathogen defense, while in basal eukaryotic invertebrates, such as the colonial ascidian *Botryllus schlosseri* and the cnidarian *Hydractinia symbiolongicarpus*, self/nonself recognition functions when individuals fuse to form vascular and hematopoietic chimeras (5). In bacteria, such as *Proteus mirabilis*, swarming colonies can recognize each other as nonself and establish a visible boundary, whereas genetically identical swarms merge (6, 7). In protists such as *Dictyostelium discoideum*, self/nonself recognition limits "self" genotypes to sporulating fruiting bodies (8, 9). In plants, self/nonself recognition is important for both pathogen defense and the prevention of self-fertilization for out-crossing species (10, 11). In filamentous fungi, the syncytial lifestyle combined with cell fusion to form the interconnected hyphal networks that are the growth habit of these organisms makes them superb models for studies of self/nonself recognition mechanisms. In this chapter, we summarize the current knowledge about somatic cell fusion events in filamentous fungi that govern both self and nonself recognition processes and that act prior to and after cell fusion events.

SOMATIC FUSION IN FILAMENTOUS FUNGI AND ITS CONSEQUENCES: BENEFITS AND DETRIMENTS

In filamentous fungi, somatic cell fusion (anastomosis) is essential to develop the hallmark of filamentous fungal growth: an interconnected, multinucleated, syncytial network (Fig. 1). Fusion has been reported in more than 73 species of filamentous fungi covering over 20 genera (12). Unlike other organisms, such as plants or animals, fungi lack biological transport networks (vascular systems). Instead, these organisms build a mycelial network to distribute cytoplasm, organelles (including nuclei), nutrients, and other resources within the syncytium, facilitating growth and rapid spatial expansion of the fungal colony (13–16). In fact, filamentous fungi form the most extensive biological networks characterized so far (17). Anastomosis in hyphae is also critical for host colonization and virulence of pathogenic and symbiotic fungi (18, 19) and for mycoparasitism (20).

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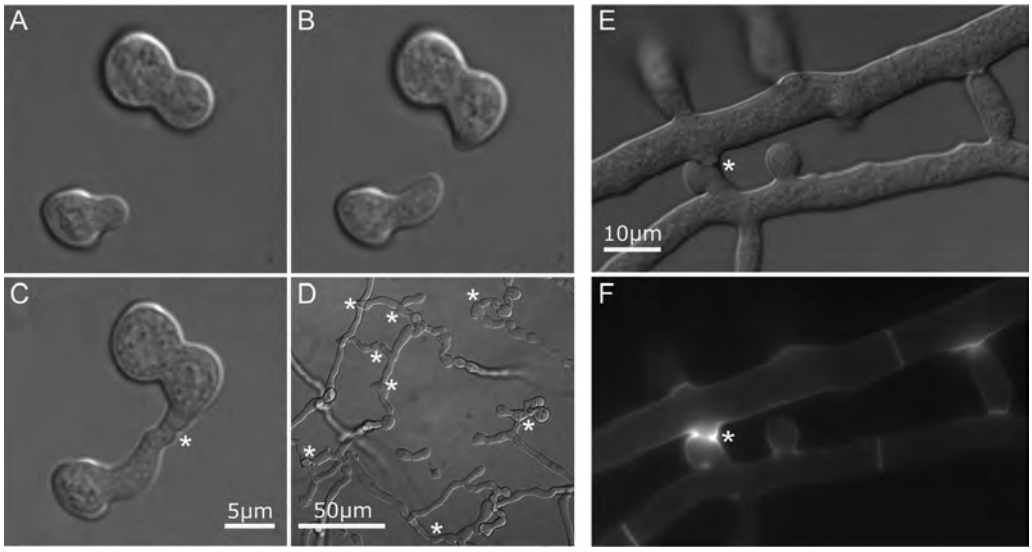


Figure 1 Germling and hyphal fusion in *Neurospora crassa*. (**A–C**) Germinating spores undergo mutual attraction and fusion (A: 0 min; B: 40 min; C: 80 min). (**D**) Consecutive fusion events result in network formation. (**E, F**) Hyphal branches fuse and form cross connections. (E: DIC; F: cell walls stained with calcofluor white). Asterisks in all images indicate fusion points. Adapted from reference 38.

In addition to fusion within a colony, cell fusion can occur between genetically different colonies. Such fusion events of nonclone individuals can be viable and lead to the coexistence of genetically different nuclei in a common cytoplasm (heterokaryon). Heterokaryon formation in filamentous fungi has potential benefits; e.g., it results in functional diploidy and mitotic recombination during the parasexual cycle, which can be especially important for highly clonal species (21, 22). However, heterokaryon formation has risks, because infectious agents can be transmitted via fusion events (23–26). Within a heterokaryon, allorecognition processes determine the fate of the fused cells: compatible genotypes lead to viable heterokaryons indistinguishable from a homokaryotic colony, while heterokaryotic cells resulting from the fusion of incompatible genotypes are rapidly compartmentalized and undergo a programmed cell death (PCD) reaction, termed heterokaryon (or vegetative) incompatibility (HI or VI) (27, 28). It has been proposed that HI limits genome exploitation and represents a defense mechanism that has important adaptive value in the highly competitive biotopes inhabited by most filamentous fungi (29). HI has been shown to limit the propagation of deleterious agents such as mycoviruses, DNA transposons, and senescence plasmids (24, 30–32). However, a recent study by Bastiaans et al. showed that fusion among fungal colonies is mutually beneficial, relative to the absence of fusion upon nonself recognition, suggesting an interplay between beneficial aspects of cell fusion versus the risk factors associated with indiscriminate fusion with other partners (33).

Unlike somatic fusion, where genetic similarity between the fusion partners is favorable, in out-crossing species of filamentous fungi, fusion between genetically dissimilar partners is necessary for sexual reproduction. In heterothallic fungi, genetic dissimilarity is determined by the existence of at least two different mating types. Interestingly, in some fungal species, mating type also serves as an HI factor during somatic fusion, preventing the existence of different mating types within a heterokaryotic colony (34, 35). In species capable of outcrossing, individuals that are somatically incompatible undergo sexual reproduction and production of meiotic progeny, indicating that nonself recognition involved in HI processes must be inactive during the sexual cycle.

RECOGNITION, CELL FUSION, AND COLONY ESTABLISHMENT

In addition to hyphal fusion within a mature colony, somatic fusion events also contribute to the onset of colony formation. For example, cell fusion between germinated asexual spores (germlings) merges numerous individuals into one functional unit, providing the basis for the developing mycelial colony in a number of fungal species (Fig. 1) (36). The ability of germlings

to form a network contributes to fitness, as indicated by more rapid colony establishment and subsequent asexual spore formation (36–39). However, fusion can be particularly risky for germlings. For example, a post-fusion incompatibility reaction (see below) would likely result in the death of both germlings. Therefore, most HI reactions described so far are suppressed at the germling stage (40).

Germlings are especially tractable for analyzing the cellular mechanisms and components involved in the cell fusion process (2). In recent years, more than 60 proteins involved in germling fusion have been identified (41, 42). Most of the cellular components mediating germling fusion processes are also required for hyphal fusion in a mature colony, indicating that both processes share common molecular machinery. Mutual recognition and attraction of the fusion partners employs an intricate signaling network, which comprises conserved eukaryotic factors, such as mitogen-activated protein kinase (MAPK) modules, NADPH oxidases, the striatin-interacting phosphatase and kinase (STRIPAK) complex, and calcium-regulated factors, but also fungal-specific proteins (Fig. 2). A central hub within this network is a MAPK cascade homologous to the pheromone response pathway of *Saccharomyces cerevisiae* (43, 44). Analysis of the subcellular dynamics of the MAPK mitogen-acivated kinase-2 (MAK-2) during germling fusion in *Neurospora crassa* revealed an unusual mode of communication associated with localization of MAK-2 to cell tips during chemotropic

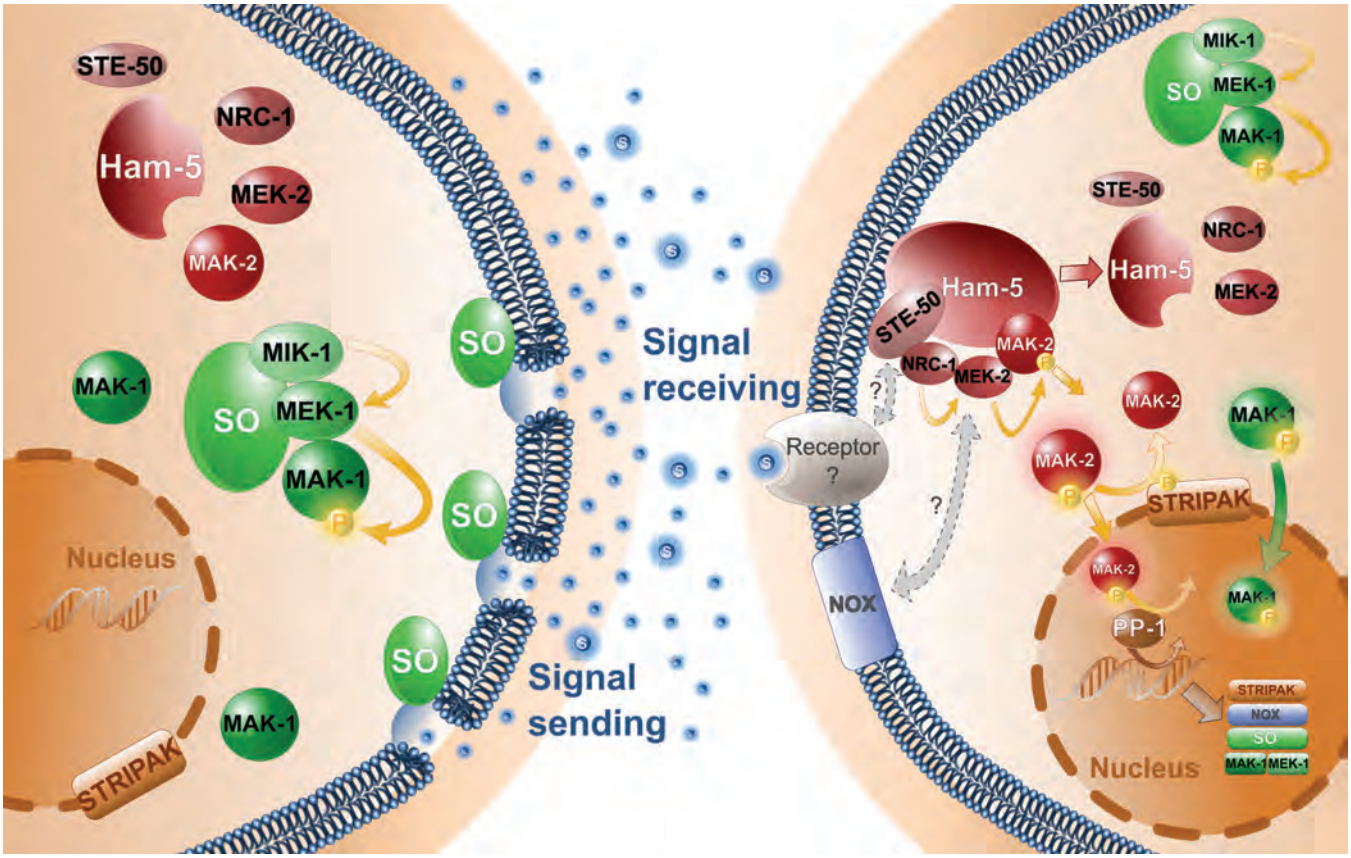


Figure 2 Working model of the molecular events governing germling and hyphal fusion. The signal-emitting cell releases the ligand in a pulse-like manner, probably by exocytosis. Binding of the signal molecules to their cognate receptors results in assembly and activation of the MAK-2 module at the plasma membrane. MAK-2 phosphorylates MOB-3 of the STRIPAK complex, thereby promoting nuclear entry of MAK-1. In the nucleus MAK-2 activates the transcription factor PP-1, which controls cell fusion factor-encoding genes. Activation of MAK-2 involves reactive oxygen species production by the NADPH oxidase (NOX) complex either upstream or downstream of the MAP kinase cascade. Adapted from reference 38.

interactions (45, 46). During the tropic interactions between germlings or hyphae, MAK-2 assembles with its two upstream kinases MEK-2 and NRC-1, the scaffold HAM-5, and the adaptor protein STE50 in an oscillating manner at the plasma membrane of cell tips (45, 47, 48). Within one cell, formation of the MAK-2 complexes at the cell tips alternates with the membrane recruitment of another protein, called SOFT (SO), a factor that is only conserved in filamentous ascomycete fungi and is essential for germling/hyphal fusion (45, 49). The membrane recruitment of the MAK-2 complex or SO alternates between the two fusion partners in a coordinated manner, with one phase lasting between 8 and 12 minutes. These observations indicate that the two fusion cells switch between two physiological states in a highly coordinated manner.

The current working model suggests that the cells take turns in signal sending and signal receiving. Mathematical modeling revealed that this unique mode of communication would allow signaling via a single receptor/ligand pair employed by both partner cells (50). The spatiotemporal coordination of signal sending versus signal receiving would enable genetically and developmentally identical cells to achieve mutual attraction and fusion, while avoiding self-stimulation. While the nature of the predicted signal and receptor activating the MAPK module remains unknown, the linkage of the MAK-2 module and the SO protein within the fusion signaling network is beginning to unfold. In *Sordaria macrospora*, a homolog to SO called PRO40 functions as a scaffold for a second MAPK module, the cell wall integrity pathway (51). Similar to the MAK-2 pathway, this MAPK module is also essential for somatic fusion in several filamentous fungi (43, 51–53). In *N. crassa*, nuclear entry of the MAPK of the cell wall integrity pathway, MAK-1, is controlled by the STRIPAK complex in a MAK-2-dependent manner (54). STRIPAK complexes are multiprotein assemblies that mediate a plethora of biological functions in eukaryotic organisms. Their presence in filamentous fungi was first shown in *S. macrospora*, and their essential role for somatic fusion has been established for several species (54–59). Besides the STRIPAK complex, an additional target of MAK-2 is the transcription factor PP-1, which in turn controls the expression of several fusion-mediating factors, including genes encoding the STRIPAK complex, *so*, *mak-1*, *mek-1*, and NADPH oxidase encoding genes (39). NADPH oxidase complexes are essential for somatic fusion in several filamentous fungi, and their potential interaction with the MAPK signaling modules as targets or activators is currently a major research topic in the cell fusion field (2, 60–62). In general, placing the numerous identified fusion factors into the described signaling network will be one of the main future challenges in this research field. Given, however, the experimental tractability of fungal model systems, hyphal and germling fusion may well advance as paradigms for studying eukaryotic signaling networks and their subcellular dynamics.

NONSELF RECOGNITION EVENTS ACTING PREFUSION

While the molecular basis of chemotropic interactions between genetically identical germlings has been studied extensively, there are only a few studies focusing on processes involved in chemotropic interactions between genetically nonidentical germlings. For example, prerecognition of incompatible strains has been reported in *Tuber borchii* and *Glomus mosseae*, in which incompatible hyphae avoid fusion with each other (63, 64). Although the genes and mechanisms behind these findings remain to be revealed, these observations suggest a link between fusion signaling and nonself recognition. This hypothesis was supported recently in a study by Heller et al. showing that the polymorphic "greenbeard" genes *doc-1*, *doc-2*, and *doc-3* mediate kind discrimination in *N. crassa* populations and act at a distance during chemotropic interactions. Kind discrimination divides *N. crassa* populations into separate communication groups; only individuals with identical sets of *doc* genes (kind individuals) show chemotropic growth and cell fusion. Germlings from different communication groups grow past each other, even when in close proximity, to find a partner of their own communication group. If nonkind germlings are in close proximity, MAK-2 oscillation reinforcement is repressed, resulting in an inability of cells to establish chemotropic interactions (65). These findings show that cellular communication in fungi is even more complex than initially anticipated: due to nonself recognition mechanisms acting at a distance, cells must avoid not only self-stimulation during chemotropic interactions, but also stimulation by nonkind individuals.

NONSELF RECOGNITION MECHANISMS ACTING POSTFUSION

In spite of nonself recognition mechanisms that act prefusion, fusion between genetically different strains occurs frequently. Anastomosis between compatible genotypes results in formation of a viable heterokaryon, while anastomosis between incompatible genotypes triggers HI, resulting in a PCD reaction leading to death of

the fusion cells (Fig. 3). This outcome is widespread in fungi and represents the most probable event after heterokaryon formation. Heterokaryon (or vegetative) incompatibility can be observed by the appearance of a demarcation line, which separates the genetically incompatible strains, termed "barrage." One of the first accounts of this type of rejection of nonself in filamentous fungi was made in the early 20th century by the pioneering mycologist Dorothy M. Cayley on the plant pathogenic ascomycete *Diaporthe perniciosa* (66). After confronting mycelia of monosporic cultures that exhibited the barrage phenotype, Cayley noted that "like must meet like and unlike shows aversion to unlike." HI reactions in nature, mostly on dead tree trunks, or on an agar plate under laboratory conditions, can be detected by assessing whether barrage lines form at interaction zones between genetically distinct individuals (Fig. 4) (67). Microscopically, incompatible heterokaryotic fusion cells are rapidly isolated from the rest of the mycelia by septal plugging (68, 69). Septal plugging in response to stress can be mediated by specialized peroxisome-derived organelles called Woronin bodies (70–73), although the role of the Woronin body in HI is unclear. However, a family of septal pore-associated proteins and the fusion protein SO have been associated with septal plugging during HI in *N. crassa* (74, 75). The HI reaction leads to vacuolization of the fusion cells accompanied by cell wall thickening, lipid droplet accumulation, reactive oxygen species production, and intracellular *de novo* formation of septa (Fig. 5) (70, 76). Furthermore, nuclear DNA degradation during cellular dismantling occurs, drawing parallels between fungal PCD and *apoptosis* in metazoans (77). The complete cellular lysis of an incompatible hyphal compartment can vary in duration, ranging from 20 minutes to more than 6 hours, depending on the genetic determinants triggering the reaction. Autophagy also plays a role in HI, although the autophagosomal process is not required for the execution of cell death (78, 79). It has been proposed that

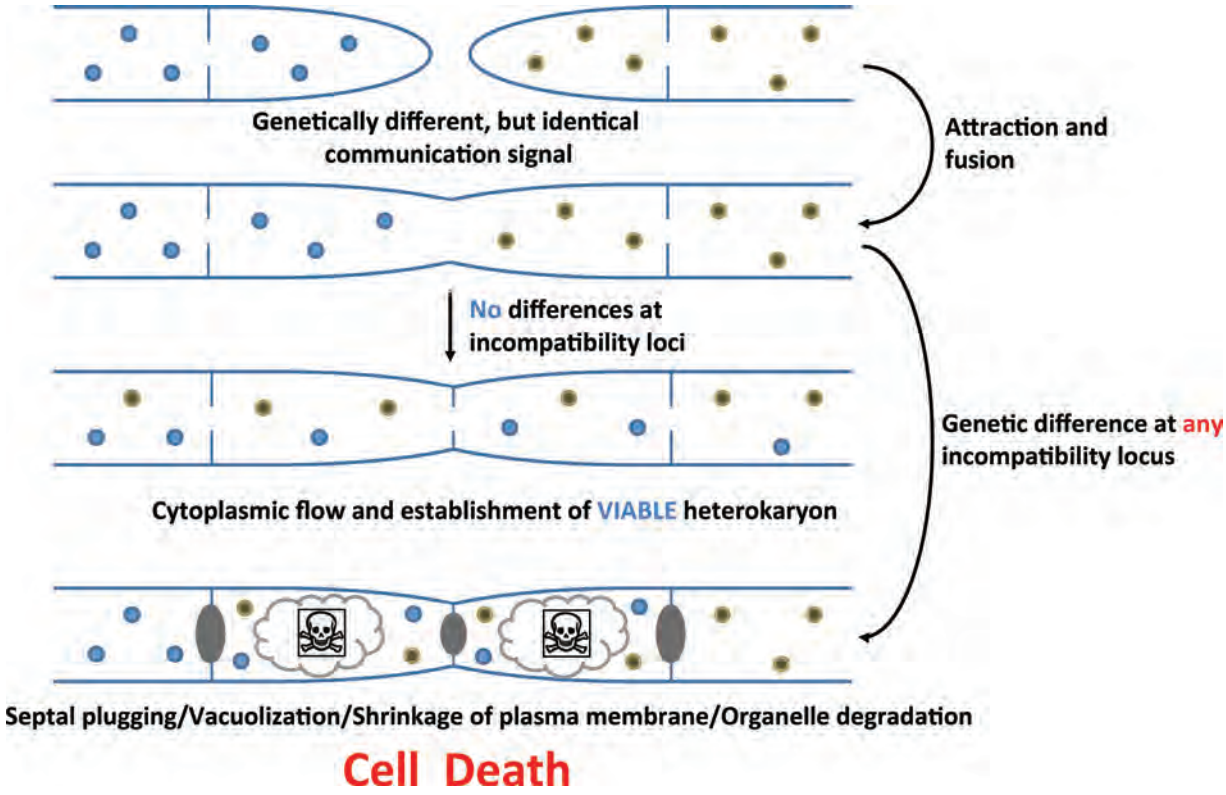


Figure 3 Heterokaryosis and its possible outcomes. Genetically distinct individuals can undergo hyphal anastomosis. If there are no allelic specificity differences at *het* loci, a viable heterokaryon is established and nuclei (blue and brown dots) are exchanged. If allelic specificity is different between the two strains for any of the *het* loci, septal plugging isolates the heterokaryotic compartments and cell death occurs.

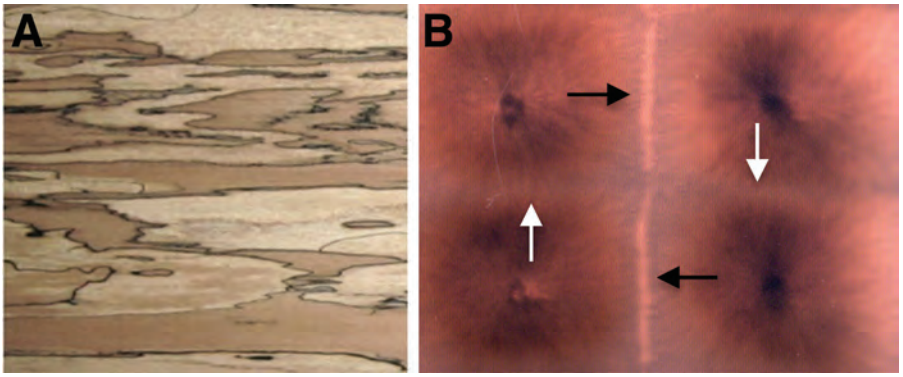


Figure 4 Macroscopic visualization of vegetative incompatibility. The heterokaryon (vegetative) incompatibility reaction is visualized by the occurrence of a demarcation line called "barrage" that separates the incompatible strains. (**A**) Evidence of barrage on wood (spalted wood) occurring in the wild. (**B**) Barrage reaction (black arrows) between genetically incompatible *Podospora anserina* strains. Identical individuals fuse without inducing allorecognition PCD and do not form the barrage (white arrows).

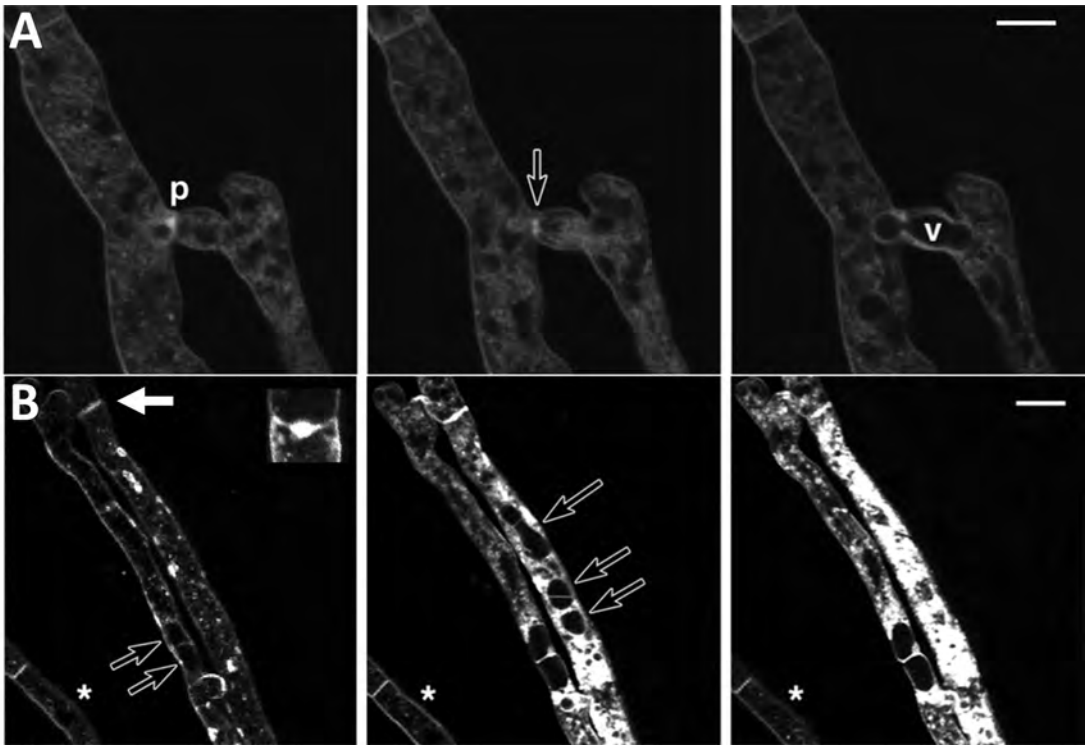


Figure 5 Microscopic visualization of programmed cell death during vegetative incompatibility. A time course of compatible and incompatible hyphal fusion in *Neurospora crassa*. The programmed cell death reaction is followed by the fluorescent vital dye (membrane staining) FM4-64. (**A**) Fusion between two *N. crassa* strains that have identical specificities at all *het* loci. Arrow shows the fusion pore (p). Nuclei or large vacuoles (v) are transported through the pore with the cytoplasmic flow. (**B**) Fusion between two *N. crassa* strains that differ in *het* specificity. Heterokaryotic cells are compartmentalized by septal plugs (solid arrow and insert). Permeabilization of the plasma membrane leads to increased cytoplasmic staining and vacuolization. Open arrows show large vacuoles within incompatible fusion cells, while the asterisk shows a nearby healthy cell. Bar = 10 μM. Adapted from reference 146.

autophagy has a protective role during HI by limiting the spread of the PCD signals, similar to what has been reported in plant immune responses (80).

Genetic Control and Molecular Characterization of Heterokaryon Incompatibility

Early efforts to understand the HI reaction led to genetic mapping of loci involved in its control for a small number of ascomycete species, so-called *het* (heterokaryon) or *vic* (vegetative incompatibility) loci (81, 82). Genetic analyses revealed that the number of *het/vic* loci ranges from 7 to 12 for different species in sampled populations. Nonself recognition in HI systems may be mediated by alternate alleles at a single *het* locus (allelic HI systems) or by alternate alleles at more than one *het* locus (nonallelic HI systems). Within the three species (*N. crassa*, *Podospora anserina*, and *Cryphonectria parasitica*) which have served as model organisms for studying HI, nonallelic HI systems predominate (Fig. 6). Independent of the type of HI system, particular hallmarks of molecular evolution are shared between *het* loci. Foremost, alleles at a particular *het* locus are highly polymorphic in populations and are often found in hypervariable genomic regions (83). Second, alleles conferring alternate specificity are maintained in wild populations in nearly equal allelic frequencies, which is an indication of nonneutral balancing selection operating on these loci (83, 84). The observed signatures of balancing selection can be explained by the associated benefits of preserving incompatible alleles and are in accordance with the proposed adaptive value of HI. The underlying evolutionary process is an example of negative frequency-dependent selection in which the rare genotype is advantageous: strains carrying a less frequent *het* allele would potentially form a higher number of incompatible heterokaryons and therefore possess a fitness advantage by diminishing conspecific parasitism (85, 86). In some cases, the evolutionary pressure on HI determinants surpasses speciation events resulting in trans-species polymorphism in which closely related species share ancestral *het* alleles. Balancing selection and trans-species polymorphisms are described for other self/nonself defining loci such as the major histocompatibility complex in vertebrates and the S locus in flowering plants (87, 88).

In the next section we summarize the molecular details for the currently characterized *het* genes and HI systems of each of the three above-mentioned fungal models.

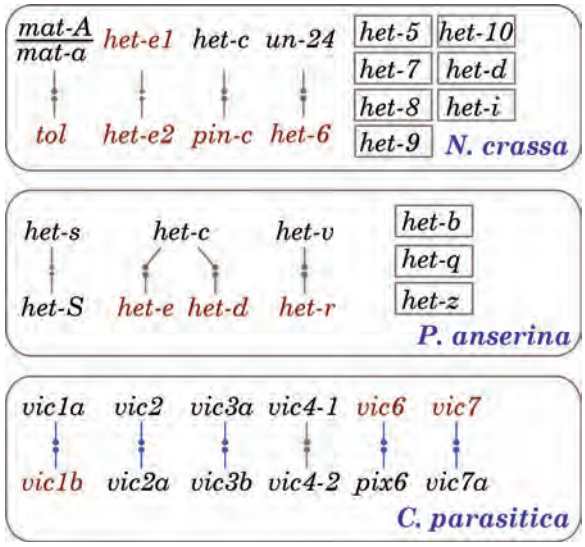


Figure 6 Incompatibility systems and genetically identified *het* (*vic*) loci in model filamentous ascomycete species. Round-headed arrows connecting the *het/vic* genes indicate nonallelic HI systems, and square-headed arrows indicate allelic HI systems. Blue arrows indicate that the incompatibility reaction influences the distribution of mycoviruses that result in hypovirulence in *Cryphonectria parasitica*. Genes in red encode for proteins with a HET domain, and boxed genes (loci) are still not identified molecularly.

N. crassa

N. crassa is a heterothallic ascomycete with a haploid cell cycle and initially was used as a model organism to establish the bases of molecular biology and decipher metabolic pathways (89, 90). Classical genetics assays (complementation or dominance/recessiveness) with haploid monokaryotic strains carrying auxotrophic mutations revealed that heterokaryosis between strains of opposite mating types results in growth inhibition (91). Incompatibility based on differences at the mating type locus is not exclusive to *N. crassa* and is found in several other unrelated species (92, 93). The mating type locus in *N. crassa* is composed of genes that confer mating identity (*mat A* or *mat a*) and are evolutionarily unrelated (termed idiomorphs) (94). The *mat-A1* and *mat-a1* genes of the *mat A* or *mat a* idiomorphs, respectively, encode for transcriptional regulators which trigger HI when coexpressed in heterokaryotic cells (34, 95). The role of mating of the *N. crassa mat* locus is uncoupled with its HI function as demonstrated by strains carrying mutations in *mat-A1* or *mat-a1* that abolish HI but that do not impact mating competency (96, 97). Additional genetic analysis for suppressors of mating type incompatibility led to the identification of a recessive mutation called *tol* (tolerant) (98).

Strains carrying the *tol* mutation mate normally but do not induce the HI phenotype. TOL actively regulates mating type-dependent HI, as shown by introgression experiments between *N. crassa* and the closely related pseudohomothallic species *Neurospora tetrasperma* (99, 100). The *tol* gene encodes for a putative 1,011-amino-acid polypeptide with a HET domain and coiled-coil and leucine-rich repeat regions. Transcriptional repression of *tol* during the sexual reproductive phase has been postulated to allow opposite mating type nuclei to coexist in the dikaryotic ascogenous hyphae (93).

Further investigations of heterokaryosis with nearly isogenic *N. crassa* strains or with the elegant use of strains containing partial chromosomal duplications revealed 10 other *het* loci in *N. crassa* (81, 101, 102). Three of these *het* genes have been cloned and their products examined at the molecular level. Two of the *het* genes, *het-c* and *het-6*, control HI in association with closely linked (adjacent) genes termed *pin-c* (partner in incompatibility of *het-c*) and *un-24*, respectively (81, 103, 104, 105). Nonallelic interactions between *het-6* and *un-24*, the latter encoding the large subunit of ribonucleotide reductase, are considered crucial for the HI phenotype in the *het-6*/*un-24* system (106). Haplotypes of *het-c*/*pin-c* and *het-6*/*un-24* are under balancing selection, show severe linkage disequilibrium—haplotypes are inherited as blocks—and exhibit trans-species polymorphism (84, 107). The product of *het-c* is a glycine-rich cell wall protein, and the HI specificity of the allelic variants is determined by indels in a variable region (108, 109). The *pin-c* gene encodes a protein that shares a region of similarity with the products of *het-6* and *tol*, termed the HET domain (~150 amino acids). Genes encoding predicted HET domain proteins are ubiquitous in ascomycete genomes (83). A HET domain protein is also encoded by the recently characterized *het-e* locus (83), and coexpression of different *het-e* alleles triggers the HI reaction. Proteins encoding a HET domain are a common thread in all molecularly characterized HI systems in *Neurospora*, suggesting downstream similarities in PCD pathways. A combined population genomics and evolutionary approach was used to identify 15 additional candidate *het* loci, all encoding proteins with a HET domain and which displayed at least two long-diverged haplogroups (83). Additionally, deletion of a transcription factor, *vib-1*, suppresses the HI reaction mediated by genetic differences at all molecularly characterized *het* loci in *N. crassa* and was shown to be required for expression of some *het* genes encoding HET domain proteins (110–112).

P. anserina

P. anserina is an ascomycete in the same order (Sordariales) as *Neurospora* (68). Eight HI systems have been genetically defined in *P. anserina* and four have been molecularly investigated (70, 82). To date, none of the characterized *het* loci in *P. anserina* are orthologous to those described in *N. crassa*. As in *N. crassa*, three nonallelic HI systems (*het-d*/*het-c*, *het-e*/*het-c*, and *het-r*/*het-v*) depend on a gene encoding a HET domain protein (*het-d*, *het-e*, and *het-r*) (113, 114). The HET domain itself has been shown to play an essential role in the induction of PCD in *P. anserina* (115). The HET domain is situated at the N-terminus of these proteins next to a central nucleotide-binding NACHT domain and a C-terminal domain composed of WD40 repeats (116). The NACHT domain is associated with regulation of apoptosis and innate immunity in metazoans and mediates the oligomerization of proteins forming multimeric cell death platforms like the apoptosome (117, 118). The WD40 repeats form a doughnut-shaped beta-propeller structure where individual repeats of 40 to 42 amino acids, in beta-sheet conformation, revolve around a central axis and can function in molecular sensing as protein scaffolds (119). APAF-1, the human apoptotic factor assembling the apoptosome, also has a WD40 domain which binds to cytochrome *c*, thus regulating its apoptosis-inducing activity (120). The NACHT and WD40 domains have been shown to be functionally important in HET-E to trigger HI (121).

The coexpression of allelic variants of HET-D or HET-E with antagonistic variants of HET-C—a protein with a GLTP (glycolipid transfer protein) fold—induces a PCD reaction (122, 123). In this nonallelic incompatibility system, the *het-c* gene is not linked to *het-e* or to *het-d* (124). Eleven highly polymorphic *het-c* alleles are present in a population of 110 *P. anserina* strains, falling in seven distinct functional classes with polymorphic positions under positive diversifying selection (high ratio of nonsynonymous to synonymous substitutions per codon) overlapping with positions defining allelic specificity (125). The allelic specificity of HET-D, HET-E, and HET-R is defined by the variability of the WD40 domain (113). Exchange between the WD40 repeats between alleles from the same *het* locus and/or between different paralogous WD40 genes is hypothesized to aid in concerted evolution of alleles (126, 127). The current data support a molecular model for *het-d*/*het-c* and *het-e*/*het-c* HI systems in which direct binding of antagonistic HET-C allelic variants by the WD40 domain of HET-D/HET-E triggers the PCD reaction (128).

One HI system in *P. anserina* that has been extensively studied involves the *het-s* locus, which encodes two functional alleles, *het-s* and *het-S* (129). Importantly, the *het-s* allele encodes a prion protein (the HET-s protein) that can exist as a soluble monomer, in a state termed [Het-s*] or as infectious aggregates (a prion state) termed [Het-s] (130, 131). The appearance of the [Het-s] prion state occurs sporadically at a low rate and converts the [Het-s*] state to the [Het-s] prion, so that strains of the *het-s* genotype are exclusively prion-infected or prion-free. Only prion-infected [Het-s] strains trigger HI with [Het-S] strains (132). The HET-S protein is a pore-forming toxin that is activated upon interaction with the HET-s prion protein, resulting in death of the cell (133, 134). The two alleles *het-s* and *het-S* are under balancing selection and in a wild population of *P. anserina*, with 92% of the strains of *het-s* genotype being prion-infected, thus having the HI competent [Het-s] state (24). These observations underline the adaptive value of the allorecognition system in the wild—a conclusion supported in the same study by the correlated distribution of a deleterious plasmid with the frequency of the *het-s* and *het-S* genotypes in populations (24).

C. parasitica

Six *vic* loci control the incompatibility reaction in *C. parasitica*, an ascomycete that causes the chestnut blight disease (135, 136). As in *Podospora*, HI in *Cryphonectria* is observed by the appearance of a barrage between incompatible strains. Moreover, a systematic exploration of the transmission of virulence-attenuating mycoviruses during heterokaryosis showed that genetic differences at five of the six *vic* loci reduced virus transmission between strains, suggesting fitness benefits associated with HI (31, 137). This approach makes *Cryphonectria* an exciting model to study the relationship between HI and its function as a barrier against mycovirus transmission. The six incompatibility systems in *Cryphonectria* are composed of closely linked polymorphic genes or idiomorphs, and their functional characterization indicates mostly non-allelic interactions (31). Three of the HI systems rely on proteins with HET domains, and one other involves a protein with NACHT-WD40 architecture. As above, the molecularly characterized *vic* genes are not orthologous to *Podospora* or *Neurospora* molecularly characterized *het* loci. Recently, as a consequence of the molecular characterization of the *vic* genes, a super mycovirus donor strain has been engineered that carries multiple deactivated *vic* genes, which could potentially serve as a more potent biocontrol agent against larger sets of wild isolates that are capable of causing the chestnut blight disease (138).

HI Determinants: at the Molecular Crossroad of Allo- and Xenorecognition

Experimental evidence supports the hypothesis that HI, an allorecognition process, has a beneficial role in nature, preventing conspecific parasitism and limiting harmful replicon propagation. However, similarities of some fungal HI-inducing determinants with plant and metazoan innate immunity factors, the latter mediating xenorecognition (nonself discrimination between different species), have prompted the idea that fungal allorecognition (HI) may originate from molecular networks with a primary role in heterospecific nonself recognition (139, 140). For example, HET-E (HET-D and HET-R), controlling HI in *P. anserina*, has a similar domain organization to APAF-1 and NLRC4 (a NOD-like receptor [NLR] protein), which are intracellular molecular sensors operating in mammalian apoptosis and innate immunity, respectively (Fig. 7) (141, 142). An extensive analysis of fungal NLR-like proteins—a protein superfamily that includes HET-E, HET-D, and HET-R from *Podospora* and VIC4-2 from *Cryphonectria*—suggests a similar role in xenorecognition for this class of proteins (143). Furthermore, fungal NLRs not directly involved in HI have been shown to regulate pore-forming toxins that function as *bona fide* HI-factors, like HET-S in *Podospora* (128, 144, 145). These data support the hypothesis regarding the relationship between allo- and xenorecognition systems in fungi and put the HI phenomenon in a broader context of fungal biotic interactions.

CONCLUSIONS AND FUTURE OUTLOOK

Cell fusion is of capital importance for the fungal life style. It is not only related to mating, but it is also important during vegetative growth, facilitating colony establishment, network formation, and heterokaryon formation. Nonself recognition mechanisms that function pre- and postfusion restrict the development of heterokaryons to cells that are more genetically similar. Genetic analyses have identified a large number of genes required for cell fusion, but many of these have not been placed in a cellular pathway, nor is it understood how the different cellular pathways are integrated to accomplish cell fusion events. Another major outstanding question is the relationship between the molecular mechanisms regulating cell fusion events versus those that regulate nonself recognition, both pre- and postfusion. Unlike alleles at loci required for cell

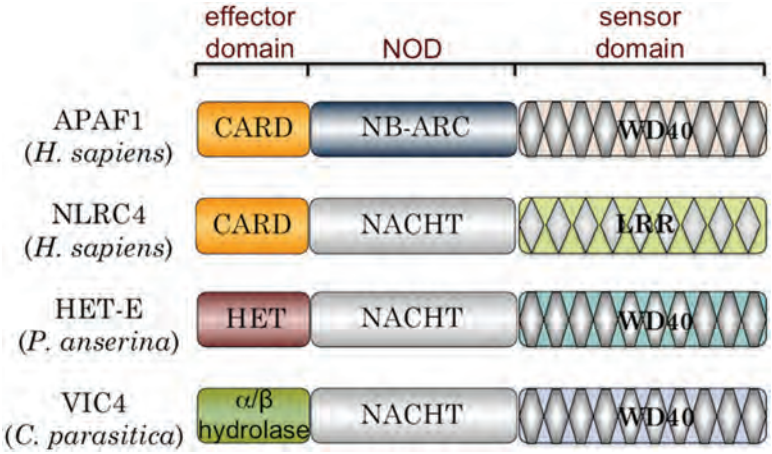


Figure 7 Domain organization of fungal and metazoan NLRs (NOD-like receptors) and NLR-like proteins. The heterokaryon determinants HET-E (also HET-D and HET-R as paralogues of HET-E) and VIC4 present a typical NLR-like domain organization. NLRs have a tripartite domain organization with a central nucleotide-binding and oligomerization (NOD) domain, an N-terminal effector domain, and a C-terminal sensor domain. The sensor domain can be composed of various repeated motifs (LRR or WD40, in the examples presented here) that trigger the activation of the receptors upon recognition of defined molecular cues. The recognition of the signal activates the formation by the receptors of multimeric protein platforms. The oligomerization of the receptors is mediated by the NOD domain (NACHT or NB-ARC type) in characterized cases, such as APAF-1 (the human apoptosis-controlling factor) and NLRC4 (an innate immunity receptor). Abbreviations: CARD, caspase recruitment domain; LRR, leucine rich repeats.

fusion, alleles at loci that function in nonself recognition often display features of balancing selection. The availability of complete fungal genome sequences on the population level will facilitate the identification and characterization of loci that display balancing selection and thus are presumptively involved in either xeno- or allorecognition.

As discussed above, the induction of PCD as a result of nonself recognition and HI seems to be conserved among species of filamentous fungi, even though the proteins used for nonself recognition can vary. However, with the exception of the *het-s/het-S* system of *P. anserina*, the link between the nonself recognition and the death reaction is still unclear for other HI systems. This raises the question of how death is induced on a molecular level. Identifying the signaling networks and exact mechanisms that control the PCD pathway will be at the center of interest for further research on fungal HI.

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Cell Biology of Hyphal Growth

11

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INTRODUCTION

Filamentous fungi are a large and ancient clade of microorganisms that occupy a broad range of ecological niches (1, 2). Fungi are recyclers, being major decomposers of plant debris (3); they form mycorrhizal symbiosis with 93% of all flowering plant families (4), and they serve in the industrial production of proteins (5). However, fungi pose a threat to public health, the ecosystem, and our food security (6, 7). The success of filamentous fungi is largely due to their elongate hypha, a chain of cells separated from each other by septa (8). Hyphae grow rapidly by polarized exocytosis at the apex (9–11), which allows the fungus to extend over long distances and invade many substrates, including soils and host tissues. Hyphal tip growth is initiated by establishment of a growth site and the subsequent maintenance of the growth axis, with transport of growth supplies, including membranes and proteins, delivered by motors along the cytoskeleton to the hyphal apex (12). Among the enzymes delivered are cell wall synthases that are exocytosed for local synthesis of the extracellular cell wall (13). Exocytosis is opposed by endocytic uptake of soluble and membrane-bound material into the cell (14). The first intracellular compartment in the endocytic pathway is the early endosomes (EEs), which emerge to perform essential additional functions as spatial organizers of the hyphal cell (15). Individual compartments within septated hyphae can communicate with each other via septal pores, which allow passage of cytoplasm or organelles (16) to help differentiation within the mycelium (17). This article introduces the reader to more detailed aspects of hyphal growth in fungi.

REGULATION OF HYPHAL MORPHOGENESIS

Hyphal morphogenesis refers to the complex biological processes that directly contribute to the formation of highly polarized hyphae by filamentous fungi. The two fundamental processes that underlie hyphal morphogenesis are polarity establishment and polarity maintenance. The regulation of these processes over time and space presumably accounts for much of the variation in hyphal morphology and growth patterns observed in the filamentous fungi (18). Additional processes that contribute to the complexity of hyphal morphology in those filamentous fungi that belong to the Dikarya include septation and the formation of septal pores (19). The regulation of these processes must also play an important role in specifying the overall organization of hyphae in these fungi. Nevertheless, we are still at an early stage in understanding the temporal and spatial regulation of hyphal morphogenesis. Progress has been achieved in determining how hyphae maintain a polarity axis (20, 21), but we still know little about how polarization sites are initially established in germinating spores and during branch formation. In addition, much remains to be learned about when and where septa are made. The following sections will address these points, but before doing so, it is perhaps useful to focus on the broader regulatory issue of how morphogenesis is coordinated with hyphal growth.

Experimentally, we typically grow filamentous fungi and assess morphogenesis under conditions that are largely homogeneous and in which all needed nutrients are available. This is unlikely to be representative of conditions that hyphae would normally experience in

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their natural environment (though there are likely to be important exceptions). In reality, most hyphae propagate in a spatially heterogeneous environment that is characterized by a patchy distribution of nutrients. Accordingly, two possible strategies could conceivably be invoked to account for hyphal extension under variable conditions. First, hyphal growth could be a "brute-force" mechanism by which a hypha harnesses the morphogenetic machinery (i.e., the cytoskeleton and vesicle trafficking machinery) and turgor pressure to plough ahead with colonization of the substrate. Extension rates and the frequency of branching may only be minimally adjusted to account for a changing environment. Alternatively, hyphae may fine-tune extension rates and morphogenesis to reflect the local environment at the tip and at incipient sites of branch formation. It seems reasonable to speculate that some combination of both strategies is deployed within a given mycelium. However, the latter strategy would seemingly rely more on the ability of hyphae to adjust the timing and location of polarization events, as well as to modulate the degree of polarity maintenance, in response to local environmental conditions. Insight into how this might occur comes from the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Both normally utilize an internal program to specify the position of localized cell surface expansion and cell wall deposition but are able to override the program in response to external signals such as mating pheromones (22). Accordingly, it is tempting to speculate that the signaling pathways that mediate growth and stress responses in filamentous fungi (e.g., TOR, PKA, HOG) likely interface with the mechanisms that temporally and spatially regulate hyphal morphogenesis. To date, little is known about how these pathways might impact hyphal morphogenesis, though there is growing evidence that they do mediate tropic responses that influence the directionality of hyphal extension (23). Characterization of the links between these pathways and the morphogenetic machinery would appear to be fertile territory for future investigation.

Spatial Regulation of Hyphal Morphogenesis

The key events underlying the spatial regulation of hyphal morphogenesis are the initial specification of a polarity axis (polarity establishment) and the subsequent stabilization of the axis (polarity maintenance). Two types of polarity establishment events are commonly observed in a typical hypha: spore polarization and branch formation (Fig. 1). In both cases, specification of a polarity axis occurs in a cell that is otherwise displaying isotropic or apolar growth (i.e., germinating spores or an existing hyphal compartment). Stabilization of the resulting axis requires the recruitment of the morphogenetic machinery to the specified site. As a result, cell surface expansion and cell wall deposition are subsequently confined to a discrete cortical site, which ultimately leads to the formation of a new hypha.

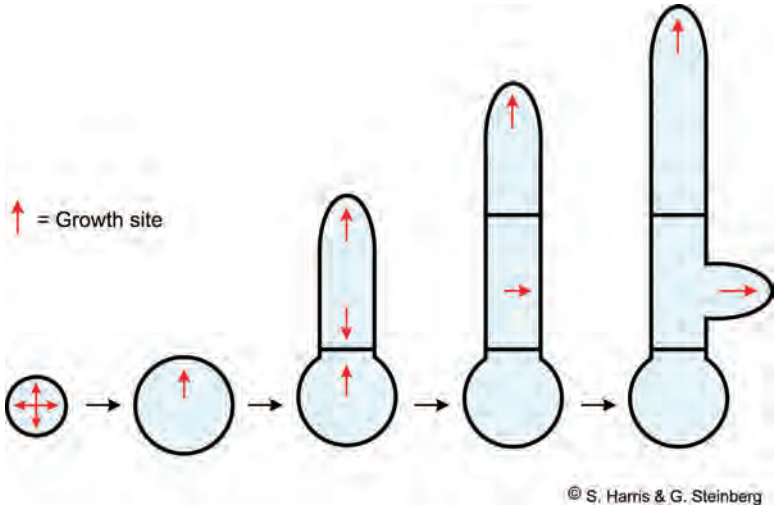


Figure 1 Growth patterns in fungal hyphae. Growth occurs in an isotropic fashion during spore germination. Specification of a polarity axis ultimately results in the formation of a hypha that continues to grow at the tip. While tip growth is maintained, the specification of additional polarity axes enables the formation of septa and lateral branches. Whereas septum formation is transient, branching results in the formation of a secondary hypha that also continues to grow at the tip. Red arrows designate polarity axes.

Detailed studies of *S. cerevisiae* and *S. pombe* have provided significant insight into the nature of the spatial landmarks that direct polarity establishment (22). *S. cerevisiae* utilizes a set of cortical landmark proteins (the Bud proteins) to specify new polarity axes (24), whereas *S. pombe* relies on microtubule-based delivery of a set of marker proteins (the Tea proteins) to position new growth sites (25). By contrast, comparatively little is known about mechanisms that specify new polarity axes in filamentous fungi. The Tea proteins are reasonably well conserved (26) and play a fundamental role in regulating the directionality of hyphal extension (see below). A subset of the Bud proteins is also present in filamentous fungi (27, 28), but instead they regulate septum formation (see below). Accordingly, at this time, the identities of any landmark/marker proteins that mediate polarity establishment in filamentous fungi remain a mystery. There is even some question as to whether a marking system is indeed necessary. Few examples of spatially biased spore polarization have been observed (e.g., reference 23), and when reported, it is in response to signals emanating from a potential plant host. Indeed, for those fungi whose spores are capable of producing multiple hyphae, it appears to be more important to ensure that the second polarity axis is opposite to the first. This type of bipolar spore polarization pattern is often observed and can be perturbed by disruption of the vesicle trafficking machinery, microtubules, and Tea marking system (29, 30).

A unique feature of filamentous fungi that enables the formation of mycelia is the ability to simultaneously sustain multiple axes of hyphal polarity. These axes result in the formation of branches, either lateral branches that emerge from subapical compartments or apical branches that form by "splitting" of the hyphal tip (31). As with spore polarization, branch formation requires the initial specification of a polarity axis followed by its stabilization. To date, the extent to which branching occurs in a defined pattern has not been fully explored. Furthermore, relatively little is known about mechanisms potentially involved in spatial regulation of branch formation in filamentous fungi. Nevertheless, key clues have emerged from a limited number of studies. For example, in *Aspergillus nidulans*, the absence of the Cdc42 GTPase or the FadA heterotrimeric G protein complex largely abolishes the formation of lateral branches and suggests signaling pathways that might be required for the specification of branch sites (32) (S. Harris, unpublished results). More intriguingly, the well-characterized septins appear to play a critical role in the specification and/or stabilization of new polarity axes at lateral branch sites (33). Elegant work using *Ashbya gossypii* suggests that the pivotal function of septins at incipient branch sites is to recognize membrane curvature (34). Lastly, evidence suggests that some fungi (e.g., *A. nidulans*, *Epichloe festucae*) actively suppress lateral branch formation from regions adjacent to hyphal tips through the localized accumulation of reactive oxygen species mediated by NADPH oxidase complexes (35, 36). This presumably ensures that growth is directed toward the tip and facilitates migration away from depleted substrate when nutrients are scarce.

A key feature that distinguishes hyphae from yeast cells is their ability to sustain polarized growth over a considerable distance. This implies that the regulatory systems that stabilize polarity axes must be much more stringent in filamentous fungi to support the long-range vectorial delivery of secretory vesicles to the hyphal tip. Although several components of the morphogenetic machinery have been implicated in the maintenance of hyphal polarity (20, 21), the nature and identity of any regulatory system that organizes the machinery and marks the hyphal tip remain elusive. It has been firmly established that spatially coupled exocytosis and endocytosis are essential for the stabilization of polarity axes (37, 38), and it has been suggested that this could reflect the need to recycle one or more cell surface markers that identify the hyphal tip (39, 40). It should also be added that a secondary role for the coupling of endocytosis with exocytosis might be to enable the spontaneous generation of local asymmetries that are then amplified to define a polarity axis if no functional marker is present. To date, the most compelling candidate for a hyphal tip marker is the *A. nidulans* TeaR protein (a homologue of the *S. pombe* mod5 cell end marker). TeaR is associated with exocytic vesicles that are transported on microtubules and delivered to the cell surface at the hyphal tip, where it mediates the local recruitment of the morphogenetic machinery (41). Recent evidence suggests that membrane-associated TeaR is rapidly dispersed at hyphal tips, only to be replenished again via microtubules (42). This result supports the existence of a dynamic feedback loop that continually establishes transient polarity axes at the hyphal tip that serve to maintain the overall direction of hyphal extension. The presence of lipid microdomains at hyphal tips and their importance in the formation of stable polarity axes raise the possibility that additional regulatory systems may operate in parallel with TeaR to mark the hyphal tip (43, 44).

Filamentous fungi exhibit tropic responses to chemical, mechanical, electrical, and other environmental stimuli (see references 45 and 46). The dynamic nature of polarity maintenance provides a satisfying explana-

tion for how the direction of hyphal extension can be rapidly reoriented in response to external signals. Moreover, it seems likely that the perception and transduction of external signals can override internally programmed polarity systems such as that mediated by TeaR. The demonstration that a chemotropic response is mediated by a cell surface pheromone receptor and a mitogen-activated protein kinase signaling pathway in the root colonizing fungus *Fusarium oxysporum* (23) provides some insight into how an existing polarity axis can be subverted by external signals.

Temporal Regulation of Hyphal Morphogenesis

In uninucleate fungal cells, the maintenance of nuclear content requires precise coordination of cellular morphogenesis with nuclear division. Mechanisms that couple these two processes have been described in some detail in *S. cerevisiae* and relatives. For example, in *S. cerevisiae*, regulatory mechanisms focused on the cyclin-dependent kinase (CDK) Cdc28 coordinate polarity transitions with nuclear division at multiple points in the cell cycle (47). *Candida albicans* is a polymorphic fungus that forms uninucleate hyphal cells, and here also, CDK plays a critical role in controlling the timing of polarity establishment, septum formation, and the transition from budding to hyphal growth (48). Key CDK targets include the septins and polarisome protein Spa2 (49, 50). The importance of CDK for the temporal coordination of morphogenesis with the cell cycle has also been documented in the plant pathogen *Ustilago maydis* (51).

In multinucleate fungal hyphae, the need to coordinate the timing of polarity establishment with nuclear division is not so apparent. Indeed, *A. nidulans* mutants incapable of nuclear division are able to form elongated hyphae (52), and mutants that fail to undergo spore polarization are able to complete multiple rounds of nuclear division (29). Despite this apparent lack of dependency, there is some evidence in *Aspergillus* species that polarity establishment and septum formation are under normal circumstances coordinated with nuclear division (29, 53, 54). This presumably reflects the need to maintain a preferred ratio of cytoplasmic volume per nucleus in growing hyphae. The underlying process, which has been termed the duplication cycle (53), remains poorly characterized, and it is not even clear whether the phenomenon is broadly conserved. For example, hyphal compartments in *Neurospora crassa* and *A. gossypii* can possess >100 asynchronously dividing nuclei, and there is no apparent evidence that hyphal morphogenesis is temporally coupled to the division of these nuclei (e.g., reference 55). One final observation potentially refutes the notion that hyphal morphogenesis is not dependent on nuclear division in filamentous fungi. In particular, the temperature-sensitive *A. nidulans nimL*, *nimM*, and *nimN* (never-in-mitosis) fail to undergo nuclear division and fail to establish polarity (unlike other *nim* mutants; references 29, 52, 56). These mutants are very sensitive to the DNA synthesis inhibitor hydroxyurea and share their polarization phenotype with wild-type conidia exposed to hydroxyurea (29). These observations suggest that completion of an as yet to be defined step in the S phase of the cell cycle might be necessary for spore polarization to proceed.

FUNGAL EXOCYTOSIS

Hyphal growth occurs by apical extension. Cell wall-modifying enzymes, substrates, and the membrane required for cell expansion are delivered to the apex by exocytosis, which suggests that the filamentous habit of growth is strongly dependent on the secretory pathway. Such an apparently simple fact has major implications both in the fungal pathogenicity field (host invasion is dependent on apical extension) and in the field of biotechnology (filamentous fungi are major producers of industrial enzymes). The hyphal mode of growth and its dependence on exocytosis represent an experimental advantage, because mutations impairing exocytosis even to a minor extent often result in reduced colony growth, making them easily scorable (57). Moreover, mutations impairing exocytic regulators delay or preclude polarity establishment (58, 59) or, when inactivated by conditional mutations in rapidly growing hyphae, often result in a tip swelling (60), which can be used as a cell-autonomous indicator of exocytic deficit.

The Golgi and Its Regulation

Although an increasing body of evidence supports the contention that unconventional protein secretion pathways do exist in fungi (61), these are relatively poorly understood. Therefore, this article will focus on the Golgi as the central hub in the conventional secretory pathway that sorts protein cargoes to their final destination, be it the extracellular milieu, the plasma membrane, or the endovacuolar system. Several recent reports testify to the interest that the mechanistic understanding of biosynthetic traffic is spawning. For brevity we will consider here reports using the genetic models *A. nidulans* and *N. crassa*. Pioneering work on motors that move carriers toward their destinations, carried out with *U. maydis*, will be considered below.

As in *S. cerevisiae*, in the filamentous ascomycetes *A. nidulans* and *N. crassa* the Golgi consists of isolated membrane-bound structures that do not pile up to form the characteristic stacks of mammalian cells (62, 63). Even though these isolated membranous structures were initially denoted Golgi "equivalents," they are *bona fide* Golgi "cisternae," equivalent to those in other eukaryotes. Their nonstacked organization represents a major experimental advantage for live microscopy studies because cisternae can be resolved by diffraction-limited optical microscopy (64–66). When imaged with fluorescent proteins, the extensively studied Golgi of *A. nidulans* is seen as a dynamic network of ring-shaped and fenestrated cisternae, often interconnected by tubular structures (64, 67, 68). This morphology is consistent with electron microscopy studies (summarized in reference 63).

The Golgi is an intrinsically transient and compositionally heterogeneous membranous entity that is constantly fed by anterograde coat protein complex II (COPII) carriers budding from transitional endoplasmic reticulum (ER) domains/ER exit sites (Fig. 2). An unanswered question in cell biology is how the protein cargo that exits the ER traffics across the Golgi apparatus and is sorted into plasma membrane- and endosome-bound carriers (69). It is widely accepted that this traffic occurs, at least in part, by cisternal maturation rather than by vesicle-mediated connections between stable cisternae. According to this view, early cisternae, which are formed by ER-derived traffic, progressively change their lipid and protein content, becoming not only gradually enriched in cargo but also, in the end, compositionally competent (see below) to break up into carrier vesicles destined for the plasma membrane and endosomes (Fig. 2). Cisternae at this final stage of maturation are denoted "late" or "TGN" cisternae (see below). The progressive attainment of late composition is thought to be mediated by retrograde COPI vesicle traffic retrieving to cisternae in earlier stages of maturation those components that do not belong to mature (i.e., later) stages (Fig. 2).

Strong support for the cisternal maturation model comes from ground-breaking studies of *S. cerevisiae* (70, 71) and from our own work in *A. nidulans* (Pantazopoulou and Peñalva, unpublished observations; see also below). However, readers should note that the maturation model very likely requires some modification to accommodate the involvement of tubular connections that are often observed between metazoan cisternae (and between fungal ones [68]). These connections could represent highways for certain types of cargo. The model also needs to accommodate the experimentally supported possibility that rapid partitioning mediated by domains of different lipid composition enables the budding of carriers at all levels (72; for overview see references 69, 71). In a useful attempt to relate the compositional and morphological variety of the Golgi with specific roles, Glick and coworkers (73) have proposed that the Golgi consists of cisternae in three different functional stages (Fig. 2). We will use this scheme for further discussion.

In stage I, denoted "cisternal assembly," ER-derived COPII carriers coalesce to form cisternae (Fig. 2). During this stage, ER protein residents that escape to the Golgi incorporated into COPII carriers recycle to their normal destination using COPI-coated retrograde vesicles (74). This is the case, for example, for TM receptors such as *A. nidulans* RerA^{Rer1}, which sorts soluble cargo at the ER into anterograde COPII carriers, and for the syntaxin SedV^{Sed5}, a key component of the SNARE machinery mediating membrane fusion at the Golgi (60, 64, 75). Membranes in stage I contain a network of peripheral proteins recruited to them by Golgi-specific GTPases that tether membranes, thereby facilitating fusion. Among them are *A. nidulans* GrhA^{Grh1} (68) and *N. crassa* USO-1 (65). As cisternae increase in size at the expense of heterotypic and homotypic fusion, they move on to functional stage II (Fig. 2).

Stage II is the glycosylation stage (73). Golgi cisternae at this stage, during which the definitive carbohydrate decoration of protein cargo is laid, are equipped with glycosidases and glycosyltransferases. Golgi glycosyltransferases extend the *O*-mannose core that is attached in the ER to a subset of cargoes, giving rise to the diverse species-specific *O*-glycans decorating fungal secreted proteins. *N*-glycosylated proteins are remodeled, after modification of core *N*-glycan structures also added at the ER, by attachment of further polysaccharide chains of variable size and composition (76). These stage II cisternae are also armed with the transporters needed to import nucleotide diphosphate-activated monosaccharide precursors from the cytosol into the cisternal lumen. GDP-mannose transporters GmtA of *A. nidulans* (77) and VRG-4 of *N. crassa* (65), homologues of budding yeast Vrg4p used in seminal studies on Golgi maturation (70), are useful to define this compartment. Cisternae at this stage no longer receive anterograde COPII carriers. However, stage II cisternae are also furnished with tethers and with the SedV^{Sed5} syntaxin to accept retrograde traffic (presumably COPI-mediated) that retrieves to them carbohydrate-modifying enzymes that escape to cisternae in a posterior stage (stage III) of maturation. The COG (conserved oligo-

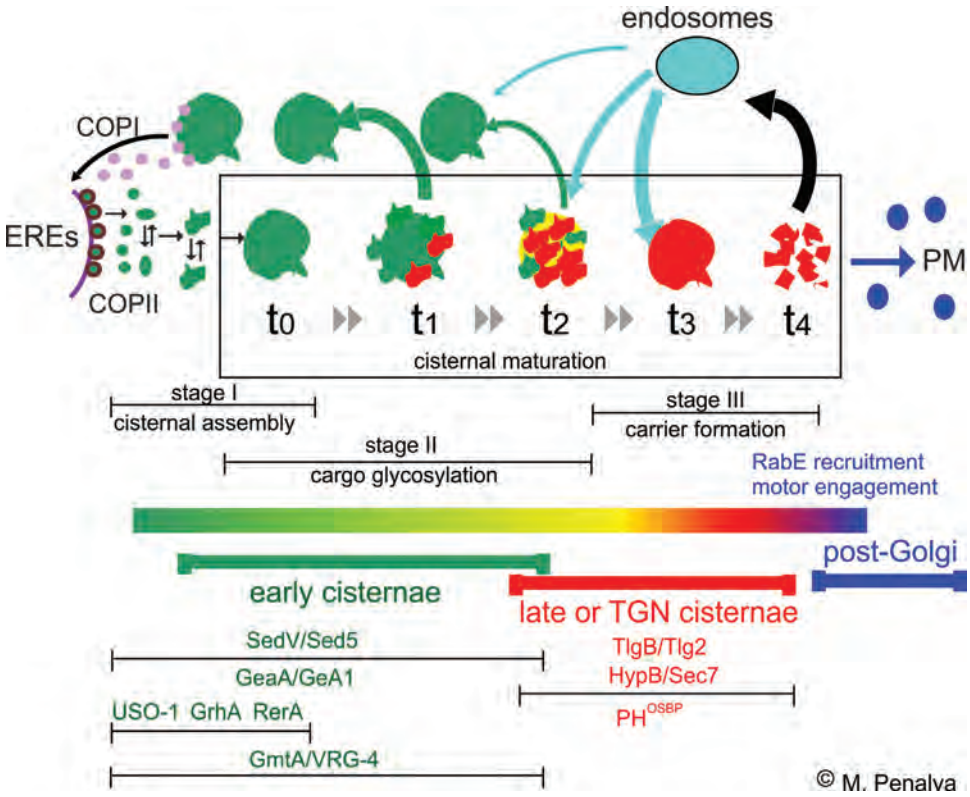


Figure 2 Highly schematic representation of the cisternal maturation process in the nonstacked fungal Golgi, with indication of the different functional stages. COPII-coated vesicles (green) bud off specialized domains of the ER denoted ER exit sites (ERES) or transitional ER (left). COPII vesicles coalesce to form an early Golgi cisterna, represented here as a green fenestrated structure that depicts actual Golgi structures often visible in EM micrographs. Retrograde COPI traffic (violet vesicles) retrieves back to the ER proteins such as cargo receptors that need to be recycled. Early cisternae are equipped with cargo glycosylation enzymes (t0). As time passes (double arrowheads) an early Golgi cisterna becomes progressively enriched in cargo and late Golgi components (represented in red) by delivering early Golgi ones (e.g., glycosylating enzymes) to cisternae in earlier stages of maturation, in a process which is likely mediated by COPI retrograde traffic (t1 and t2). Eventually, late Golgi components become predominate (TGN, t3) and the cargo-enriched cisterna becomes competent to tear off into carriers destined for the plasma membrane (PM) and the endosomes (t4). TGN cisternae also receive traffic from the endosomal system (blue arrows). In the route (dark blue) connecting the cisternae with the PM, the transition between late Golgi and post-Golgi identity is dictated by the recruitment of $RabE^{RAB11}$ to the membranes, which is critically regulated by TRAPPII (see text). Proteins that have been shown by microscopy to localize to specific stages are indicated, with green lettering indicating early Golgi and red lettering indicating TGN. The image summarizes work performed with *A. nidulans*.

meric Golgi) complex (78) also mediates the tethering of these intra-Golgi carriers to stage II cisternae, such that deficient COG function results in glycosylation defects (79). The physiological importance of two *A. nidulans* Golgi COG components, $PodB^{Cog2}$ and $SwoP^{Cog4}$, has been demonstrated by showing that two classical *ts* alleles, *podB1* (polarity defective) and *swoP1* (swollen cell), cause polarization, cell wall, and protein glycosylation defects (80). The role of GmtA was established with the hyperbranching and hypomannosylating *ts* mutation *calI11* (i.e., *gmtA11*), which results in calcofluor white sensitivity (77).

Cisternae that progress to stage III, denoted "carrier forming" (73), acquire a composition that enables them to generate anterograde carriers destined for the plasma membrane and the endosomes (Fig. 2) but that incapacitates them to generate retrograde COPI traffic destined for cisternae at earlier stages of maturation.

Cisternae in this carrier-forming stage have been denoted the *trans*-Golgi network (TGN), by analogy to the most plasma membrane-proximal (*trans*) cisternae of the mammalian Golgi stacks. For all fungal purposes TGN is synonymous with "late Golgi." Traffic exiting the TGN toward endosomes is mediated by clathrin-coated carriers that use AP-1, GGA (Golgi-localized, gamma adaptin ear-containing, ARF-binding), and epsin adaptors to sort appropriate cargo into the clathrin cage. In contrast, as discussed below, carriers connecting the TGN with the plasma membrane are atypical in that they do not appear to involve any protein coat. TGN membranes contain PtdIns4*P* and the late-Golgi Arf1 GEF (guanine nucleotide exchange factor) Sec7, which has been intensively studied in *A. nidulans* (60, 81–84) and used as a prototypic TGN marker in both *A. nidulans* (68) and *N. crassa* (65). However, the most robust marker of the TGN is a fusion protein between mRFP and the PH domain of the human oxysterol binding protein (PH^{OSBP}). This chimera is recruited very efficiently and specifically to TGN cisternae by coincidence detection of active (i.e., GTP-loaded and membrane bound) Arf1 and PtdIns4*P* (85).

The cisternal maturation model implies that TGN cisternae must dissipate as they break up into transport vesicles. A study of *A. nidulans* documented the process by which TGN cisternae give rise to post-Golgi carriers destined for the apical plasma membrane (82). During this process, cisternal membranes progressively lose Golgi identity (i.e., they lose PH^{OSBP} [i.e., Arf1/PtdIns4*P*] and $HypB^{Sec7}$ [the late Golgi Arf1 GEF]) as they become increasingly rich in $RabE^{RAB11}$ (Ypt31/32p in budding yeast), the determinant of post-Golgi identity (82). By the time $RabE^{RAB11}$ peaks at a maternal cisterna, the signal of Sec7/PH^{OSBP} has virtually disappeared. At this point, $RabE^{RAB11}$-rich membranes undergo rapid movement to the Spitzenkörper (SPK), using kinesin and myosin-5 motors that power their long-distance transport to the SPK (82), consistent with a model in which $RabE^{RAB11}$ participates directly or indirectly in the recruitment of the motors (Fig. 2). The fact that cisternae that have acquired $RabE^{RAB11}$ and undergone movement toward the apex leave little signal of TGN markers behind strongly suggests that TGN cisternae largely dissipate into $RabE^{RAB11}$ exocytic carriers, which represents strong evidence for a maturation-based mechanism determining the biogenesis of these carriers (82). The biogenesis of carriers destined for endosomes, which had been filmed in *S. cerevisiae* (86), has been recently documented in *A. nidulans* (87).

Unlike early Golgi cisternae (stages I and II), TGN cisternae contain the t-SNARE $TlgB^{Tlg2}$, which colocalizes with PH^{OSBP} (60). $TlgB^{Tlg2}$ is a Qa syntaxin that forms SNARE bundles with Vti1 as Qb-, $TlgA^{Tlg1}$ as Qc-, and SynA as R-SNARE (75). In all likelihood, this SNARE bundle mediates the fusion of retrograde traffic connecting the earliest endosomal compartment, to which SynA arrives by endocytosis, with the TGN. This is the pathway that is followed by proteins that, like SynA itself, are efficiently taken up by the subapical endocytic ring and pass through the TGN before returning by way of apex-directed exocytosis to the plasma membrane (37, 64, 88). This earliest endosome, which for convenience is designated here as a "sorting endosome," should not be confused with the EEs, which are readily identifiable by their high motility on microtubule and the presence of the GTPase Rab5 (see below).

The SynA pathway is the one that must be responsible for the strong labeling of the SPK with the endocytic tracer FM4-64 (89). Another passenger of this pathway is the *A. nidulans* phospholipid flippase $DnfA^{Dnf1}$ (39). *cis*-acting mutations abolishing endocytosis of SynA (64) or $DnfA^{Dnf1}$ (39) result in a uniform rather than polarized distribution of the cargo, showing that rapid endocytic uptake/recycling combined with slow diffusion across the plane of the membrane generates polarity (90). In *A. nidulans*, traffic of cargoes between the sorting endosome and the TGN requires RAB6 ($RabC^{RAB6}$) (64) and its effector Vps52 (39). Another flippase, $DnfB^{Drs2}$, is a TGN resident that partially localizes, like $DnfA^{Dnf1}$, to the SPK. Remarkably, $DnfA^{Dnf1}$ and $DnfB^{Drs2}$ reside in different regions of the SPK (39). The role of flippases in exocytosis is not understood, but it may have major practical implications: ablation of the *Magnaporthe grisea APT2* gene (homologue of $DnfB^{Drs2}$) does not result in hyphal growth or sporulation defects but precludes the secretion of several extracellular enzymes and, importantly, prevents pathogenicity (91).

$TlgB^{Tlg2}$ is notable in that its genetic ablation is inconsequential for growth and results in a minor effect on SynA localization (75). However, *tlgB* is synthetically lethal when combined with a hypomorphic mutation in *sedV*, the gene encoding the early Golgi syntaxin (75). This indicates that the Golgi can be organized with only one syntaxin ($SedV^{Sed5}$), provided that this syntaxin is fully functional. A plausible explanation is that when $TlgB^{Tlg2}$ is absent, proteins can recycle from sorting endosomes to early Golgi cisternae in a $SedV^{Sed5}$-dependent manner and then move forward to the TGN by cisternal maturation. This interpretation is supported by the observation that $SedV^{Sed5}$, like $TlgB^{Tlg2}$, can form SNARE bundles with SynA, Vti1, and $TlgA^{Tlg1}$ (75). If this interpretation were correct, the only role of $TlgB^{Tlg2}$ would be in the fusion of

retrograde carriers derived from the endosomes with the TGN.

The Master Regulators of the Golgi: RABs, Arf1, and Their Regulators

The budding from the ER of COPII carriers that feed the Golgi, the different stages of Golgi maturation, and the exit of carriers from the TGN are governed by small GTPases of the ARF and RAB families. SarA^{Sar1} is an ER-located ARF that plays a fundamental role in the budding of COPII carriers (92, 93). Temperature shift experiments with *A. nidulans sarA^{ts}* mutations have been used to impair ER exit. As predicted by the cisternal maturation model, in cells shifted to the restrictive temperature, early Golgi residents such as SedV^{Sed5}, which have access to COPI-mediated retrograde traffic, rapidly relocalize to the ER, consistent with their Golgi localization being determined by an equilibrium between anterograde and retrograde traffic. In contrast, the TGN (late Golgi) resident TlgB^{Tlg2} does not rapidly relocalize to the ER, relocalizing instead to a diffuse cytosolic haze (92). This different behavior of early and late Golgi membranes (SedV^{Sed5} and TlgB^{Tlg2} are integral membrane proteins) is consistent with the cisternal maturation model. Notably, prolonged impairment of ER exit by *sarA^{ts}* mutations results in the formation of apical balloons surrounded by a thick wall of chitin. One possible explanation is that chitin biosynthetic enzymes are less dependent on SarA^{Sar1} function than other cell wall biosynthetic activities (92).

ARF1 (ArfA in *A. nidulans*) is an essential regulator of the Golgi (94) that plays different roles at the levels of the early and late cisternae. Two essential ArfA GEFs, GeaA and HypB^{Sec7}, localizing to the early and late Golgi membranes, respectively, ultimately mediate these roles (81). A major (and as yet unexplained) finding that illustrates the power of random classical genetic screens was that *geaA1* resulting in a single Y1022C substitution within a conserved domain of GeaA makes vegetative growth independent of HypB^{Sec7} and thus of the TGN (81). Remarkably, besides the early Golgi, GeaA^{Y1022C} localizes to the apical plasma membrane, suggesting that under these conditions early Golgi membranes can undergo exocytosis.

Three RAB GTPases are needed to organize the Golgi: RAB1 (RabE^{RAB11}, YPT-31), RAB6 (RabC^{RAB6}), and RAB11 (RabE^{RAB11}, YPT-31). RabO^{RAB1}/YPT-1 (60, 65) and RabC^{Rab6} (64) are present in both the early and the late Golgi. Notably, the three Golgi RABs are present also in the SPK (see below).

In *A. nidulans*, RabO^{RAB1} and RabE^{RAB11} are essential (60, 82). The acute inactivation of RabO^{RAB1} rapidly disorganizes both the early and the late Golgi cisternae (60). In addition to exocytosis, *A. nidulans* RabO^{RAB1} plays a crucial role in autophagy (84). The absence of RabC^{RAB6}, albeit not lethal, is severely debilitating and leads to fragmented cisternae (64). It results in delocalization of the VpsT^{VPS10} vacuolar hydrolase receptor, which recycles between the endosomes and the Golgi (64), an observation consistent with the role generally attributed to RAB6 homologues as key regulators of retrograde traffic in the secretory pathway.

RAB11 has been studied in detail in *A. nidulans* (82, 83). As noted above, RabE^{RAB11} is not a Golgi resident, but it is instead recruited to TGN membranes to determine their acquisition of post-Golgi identity. This recruitment must be governed by its GEF, which is therefore crucial for exocytosis. A long-standing question in the literature concerned the identity of the RabE^{RAB11}/Ypt31 GEF and the roles of the TRAPP (oligomeric complex transport protein particle) as a Golgi organizer. TRAPP has been isolated in three versions. TRAPPI (composed of Trs33, Ber3, Bet5, Trs23, Trs31, and Trs20 subunits) is a demonstrated GEF for Ypt1 (yeast RAB1), acting in the ER/early Golgi interphase. TRAPPIII is TRAPPI plus Trs85 and acts as GEF for Ypt1 in the preautophagosome. TRAPPII is TRAPPI plus three additional subunits: Trs120, Trs130, and Trs65. TRAPPII had been proposed to be the Ypt31 GEF acting in the TGN (95), but this role was disputed by others, who concluded that TRAPPII was the late Golgi version of the GEF that activates Ypt1 in early Golgi (96). *hypA1* is a *ts* mutation in the gene encoding *A. nidulans* Trs120 (97). The characterization of extragenic suppressors of *hypA1* combined with biochemical assays established that the physiological substrate of TRAPPII is RabE^{RAB11} (the Ypt31 orthologue). TRAPPII, as visualized with Trs120-GFP, peaks at TGN cisternae preceding their dissipation into carriers, thereby determining the Golgi to post-Golgi transition by governing RabE^{RAB11} recruitment (83). It remains to be established whether TRAPPII is assembled *de novo* in the TGN. Alternatively, TRAPPII might be assembled using as a scaffold the TRAPPI complex already present in the early cisternae that would arrive at the TGN by maturation. If this were the case, anterograde traffic across the Golgi might be mediated by a RAB1 to RAB11 cascade governed by maturation of TRAPPI into TRAPPII (95).

The Fungal SPK and Delivery of Cell Wall-Building Enzymes

The SPK is a conspicuous apical body observed at the growing apices of hyphae in fungal species belonging to the subkingdom Dikarya (Ascomycota and Basidio-

mycota). The SPK was first discovered in fixed cells of the basidiomycetes *Coprinus sterquilinus* and *Coprinus narcoticus* stained with iron-hematoxylin (10). It was later observed in live cells of *Polystictus versicolor*, where it was shown to be important for maintaining hyphal tip elongation, morphology, and growth direction (98, 99). The subsequent characterization of the SPK in diverse fungal species by phase-contrast and transmission electron microscopy identified up to nine SPK patterns (100). Mating projections of *S. cerevisiae*, mating projections and invasive pseudohyphae of *C. albicans*, dikaryotic hyphae of *U. maydis*, and germ tubes of *Uromyces phaseoli* and *Puccinia graminis* display an apical cluster referred to as an SPK-like structure thought to operate as the SPK in hyphae (101–103).

Among the non-Dikarya fungi, only *Allomyces* and *Basidiobolus* have been found to display an SPK (104, 105). Early studies reported the absence of an SPK in the Zygomycetes (106). More recently, several zygomycetous fungi were analyzed (*Gilbertella persicaria*, *Phycomyces blakesleanus*, *Rhizopus oryzae*, *Coemansia reversa*, *Mucor indicus*, *Gigaspora* spp., and *Mortierella verticillata*), and it was shown that they present instead an apical vesicle crescent (13, 107, 108). The apical vesicle crescent corresponds mainly to an accumulation of apical vesicles of different sizes, as for the SPK. However, practically nothing is known about the composition or regulation of the apical vesicle crescent vesicles. In contrast, extensive studies conducted on ascomycetous species have made it possible to identify the main components of the SPK (see below).

Hyphal growth occurs at the apices (10). Autoradiographic analysis of *M. indicus* (formerly *rouxii*) showed accumulation of tritiated *N*-acetyl-D-glucosamine (GlycNAc), a monomeric unit of chitin, preferentially at hyphal tips, indicating that apical growth was correlated to a polarized mechanism of cell wall assembly (9). Several authors proposed that this assembly of cell wall at the apex must include the participation of synthetic as well as lytic (plasticizing, softening) activities (109–111). By contrast, an alternative model not involving lytic activity proposed a plastic cell wall at the apex that would become rigid at the subapex, where remodeling glycosyltransferases would cross-link the cell wall material and rigidify it (11). Recent work with *N. crassa* has revealed two glycosyltransferases belonging to the CAZy family GH17 localized at the plasma membrane of the hyphal dome, excluding the foremost apical region immediately across from the SPK (112) (Fig. 3), in support of cell wall remodeling occurring at the subapex.

At the ultrastructural level the SPK corresponds to an aggregation of vesicles, actin microfilaments, and ribosomes and an amorphous unidentified material (113, 114) (Fig. 3). A computer simulation exercise led to the formulation of the vesicle supply center model for fungal morphogenesis (115). This model proposed that the SPK behaves as a vesicle supply center, from which vesicles travel to and fuse with the apical plasma membrane, providing the enzymatic machinery needed for cell wall expansion. Once inserted in the plasma membrane, (i) chitin synthases take *N*-acetyl glucosamine subunits (GlcNac) from the cytoplasmic side and incorporate them into a growing chain of chitin microfibrils (β-1,4-GlcNAc), and (ii) the glucan synthase takes glucose subunits (Glc) from the cytoplasmic side and incorporates them into an extending chain of β-1,3-glucan (116, 117). The sequencing of fungal genomes allowed the construction of translational fusions of specific genes with fluorescent proteins enlightening many components of the SPK.

The ultimate evidence for the suggested link between the SPK and cell wall assembly at tips was confirmed when cell wall biosynthetic enzymes such as chitin and glucan synthases were identified at the SPK in *N. crassa* (118, 119). Significantly, coexpression of differentially fluorescently tagged enzymes identified chitin synthases and components of the glucan synthase complex at the core and at the outer layer of the SPK, respectively, coinciding with the distribution of microvesicles (chitosomes) and macrovesicles reported at the transmission electron microscopy level (119, 120) (Fig. 3). Small GTPases YPT-1/RabO (Rab1) and YPT-31/RabC (Rab6) have been found occupying the inner and the peripheral layers of the SPK in *N. crassa* and *A. nidulans*, respectively (64, 65). All the above suggests different populations of vesicles containing different cell wall synthesizing activities and being regulated by different molecular switches. We are only starting to discern the lipid composition of membranes and its role in establishing membrane asymmetry and in vesicle traffic. In *A. nidulans* flippases DnfA (Dnf1) and DnfB (Drs2) also occupy distinct strata of the SPK, in support of existing different populations of vesicles (39, 121).

The SPK has been suggested to be a transfer station from cytoplasmic microtubules to actin microfilaments (122, 123). Cytoplasmic microtubules which extend through the cytoplasm and reach the SPK region are thought to participate in the long-distance traffic of vesicles to the SPK, whereas the actin cytoskeleton, found at the SPK core would mediate the flow of vesicles to and from the SPK (124, 125) (Fig. 3). In *A. nidulans* the microtubular and actin cytoskeletons are

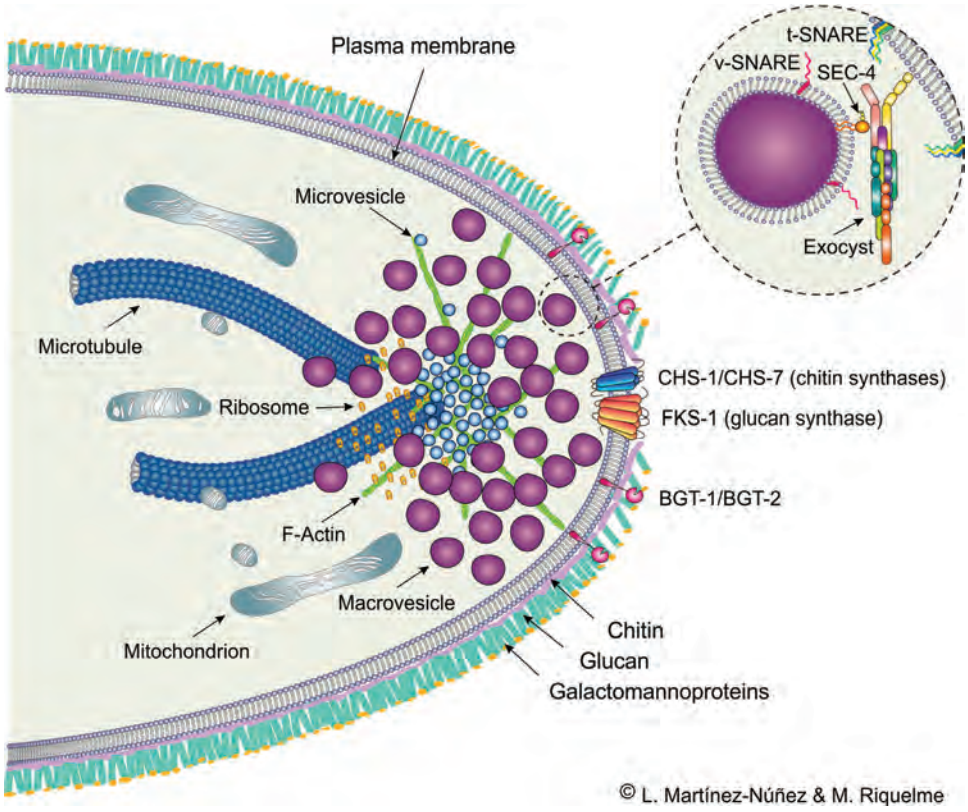


Figure 3 Illustration of a hyphal tip with the main organelles and subcellular components involved in apical cell wall growth. The diagram is based on work with *N. crassa*. (Art: Leonora Martínez-Núñez).

connected at the hyphal tips by cell-end markers (26). TeaA is delivered by microtubules to the tip plasma membrane, where it interacts with TeaR and other components, subsequently leading to the recruitment of the formin SepA, which polymerizes actin cables (126). Interestingly, while cytoplasmic microtubules are important to maintain SPK stability, hyphal morphology, and fast growth rates, they seem dispensable for vesicles to reach the SPK (127, 128).

We lack information on the biogenesis of the vesicles that constitute the SPK. While several fungal organelles have been characterized, we lack information about the post-ER and/or post-Golgi processes relative to the formation and traffic of vesicles that concentrate at the SPK (see above). It has been shown that the exocyst complex has a role in determining the site at the plasma membrane for delivery of cell wall-synthesizing enzymes (129). In *N. crassa* the exocyst components were found at two locations within the hyphal tip: at the apical plasma membrane and at the frontal region of the SPK outer layer. It is well established that an intact SPK is needed to maintain hyphal morphology and growth directionality in ascomycete fungi (130). Recently, it was found that only the aggregation of macrovesicles (not of the microvesicles) at the SPK is dependent on an intact exocyst (120). Finally, it is worth noting that the lipophilic dye FM4-64 stains the SPK in living hyphae (89). FM4-64 is taken up by the cell via endocytosis at the plasma membrane (131) (for more details see below), suggesting a connection between the exo- and endocytosis.

Myosin-Chitin Synthases in Apical Exocytosis

Our knowledge concerning the delivery of vesicles and enzymes to the hyphal tip is largely based on intensive studies of *N. crassa* and *A. nidulans*. Both organisms belong to the phylum Ascomycota, which accounts for ~64% of all fungi (2). The second major group is the basidiomycetes, which provide 34% of all described species (2). Despite the economic and ecological importance of this group, our understanding of secretion pathways in basidiomycetes is restricted to the plant pathogen *U. maydis*. In hyphae of *U. maydis*, microtubules are required to deliver chitin synthase-containing

vesicles to the apical growth region (132). *U. maydis* does not contain a typical SPK, comprised of macro- and microvesicles, but similar to *N. crassa* and *A. nidulans*, vesicles accumulate near the hyphal tip in this fungus (103). How this vesicle cluster is formed is not fully understood, but class V chitin synthases may participate in this process. Class V chitin synthases are fungal-specific cell wall-forming enzymes that carry an N-terminal myosin motor domain. This domain is even found in ancient Cryptomycota (133), suggesting that it is a hallmark of the Fungi. It was shown that the myosin motor domain of the *U. maydis* class V chitin synthase (Mcs1) (134) is not involved in apical delivery of the enzyme. Instead, this is achieved by coordinated activity of the motor proteins kinesin-1 along microtubules and myosin-5 along filamentous actin (135); the myosin-motor domain of the class V chitin synthase tethers delivered vesicles to apical actin. The transient interaction of the motor and actin increases the pausing time of the vesicles at the hyphal apex, thereby supporting polar exocytosis (135).

Interestingly, Mcs1-tethered vesicles also carry a class VII chitin synthase and a β-glucan synthase (a homologue of glucan synthase Fks1 in *N. crassa*) (136). After exocytosis, these cell wall enzymes are immobilized by newly formed polysaccharide chains, suggesting that codelivery and coexocytosis help the coordination of enzymatic activity in building the complex fungal cell wall. Myosin-chitin synthases have also been described in numerous ascomycetes, including *A. nidulans*, (137), *Aspergillus fumigatus* (138, 139), *F. oxysporum* (140), *Colletotrichum graminicola* (141), *Magnaporthe oryzae* (142), and *N. crassa* (143). Ascomycete myosin-chitin synthases have been shown to be required for morphogenesis, development, and pathogenicity (140, 141, 143, 144). As in *U. maydis*, kinesin-1 delivers *A. nidulans* myosin-chitin synthase along microtubules to the hyphal apex (145), where its myosin motor domain interacts with F-actin (146). However, whether ascomycete myosin-chitin synthases act as vesicle tethers at sites of apical exocytosis remains to be determined.

FUNGAL ENDOCYTOSIS AND EE MOTILITY

Fungal Endocytosis

Hyphal tip growth has been described as being due to the tip-ward delivery and the apical release of Golgi-derived vesicles (11, 122). In animal cells, this release (i.e., exocytosis) is balanced by the uptake of material into vesicles that form at the plasma membrane (i.e., endocytosis). These vesicles then fuse with early endosomes, and the contents can be recycled back to the surface or dispatched to the lysosome (i.e., the fungal vacuole) for degradation. For many years, endocytosis was thought to be absent in fungi (147). However, over the past 2 decades, fungal endocytosis has been recognized to be pivotal for cell function. The initial breakthrough came with the use of the lipophilic marker dye FM4-64 in the 1990s in the yeast *S. cerevisiae* (131) and then in the filamentous fungi *Uromyces fabae* (148) and *U. maydis* (149). This fluorescent dye inserts into the plasma membrane from where it is internalized by the formation of endocytic transport vesicles (131). Subsequently, studies of *M. grisea* (150), *A. nidulans* (151), *N. crassa*, and six additional fungi (89) confirmed the endocytic uptake of FM4-64 into fungal cells. These results were complemented by live-cell imaging and mutants studies in numerous fungi, including *U. maydis*, *A. oryzae*, *A. nidulans*, *C. albicans*, *A. gossypii*, and *N. crassa* (37–39, 152–160). Collectively, these studies revealed a critical role for endocytosis and endocytic recycling in hyphal growth of filamentous fungi (for overview see references 15, 57).

The Molecular Machinery for EE Motility

Fungal EEs were first described in the dimorphic fungus *U. maydis* (160). In this fungus, shortly after internalization, the dye FM4-64 concentrates in small, rapidly moving vesicular organelles (160). A putative t-SNARE, Yup1, binds these organelles via a PHOX-domain (i.e., phosphoinositide binding domain) (160, 161) that interacts with the lipid PIP3, characteristic of EEs (reference 162). Consistent with this, the FM4-64-stained organelles carry the small GTPases Rab4 and Rab5 (155, 161), both shown to reside on EEs in mammalian cells (163, 164). Thus, there is little doubt that the rapidly moving FM4-64-positive vesicles are EEs. Rab5-positive EEs were also described in *A. nidulans* (88), *N. crassa* (165), and *Zymoseptoria tritici* (166) but are not found in the yeast *S. cerevisiae*. Thus, motile EEs are a hallmark of filamentous fungi.

Microtubules are biopolymers of tubulin-dimers that support long-distance transport of organelles and vesicles in fungal cells (12, 167). They elongate at their plus end, while the minus end is usually embedded in a nucleation site, such as the fungal spindle pole body. Motility along microtubules is mediated by molecular motors, which use the polarity of microtubules to transport their cargo. In general, kinesins are known to move toward the plus end of a microtubule, while cytoplasmic dynein motors are minus end-directed. Both types of motors are "mechano-enzymes" that hydrolyze ATP to power a conformational change in the motor

domain. Repeating cycles of ATP hydrolysis result in a sequence of ~8-nm steps along the microtubule (168, 169). In filamentous fungi, the first membrane trafficking motors identified were the cytoplasmic dyneins in *N. crassa* (170) and *A. nidulans* (171), which were recognized to play a role in nuclear migration. Shortly after, kinesin motors were identified in *N. crassa* (172), *U. maydis* (149), *Nectria haematococca* (173), and the zygomycete *Syncephalastrum racemosum* (174). Interestingly, all kinesins belong to the same subgroup of kinesin motors (kinesin-1), which is thought to support tip-ward transport of fungal secretory vesicles (135) and to deliver dynein to microtubule plus ends (175, 176) (for more details on general motor function see references 12, 167 and 177). The first insight into the machinery of EE motility was provided by studies of *U. maydis*, where kinesin-3 was identified to be the motor for plus end-directed motility of EEs (178). A similar role of kinesin-3 in EE motility was discovered in *A. nidulans* (179, 180) and *N. crassa* (165). Kinesin-3 motor proteins comprise 8 of the 45 kinesins in the human genome (181) but are absent from *S. cerevisiae*. This emphasizes the similarity of membrane trafficking in filamentous fungi and elongated mammalian cells such as neurons (167, 182).

Fungal EEs have been shown to move in a bidirectional fashion along microtubules (88, 175, 183), suggesting that plus end-directed kinesin-3 gets opposed by minus end-directed cytoplasmic dynein. Indeed, deletion of the gene for kinesin-3 resulted in the accumulation of EEs at microtubule minus ends (178), whereas inactivation of cytoplasmic dynein results in a cluster of EEs at microtubule plus ends in the hyphal apex (175, 179). Interestingly, the deletion of kinesin-1 also led to EE clustering at the hyphal tip. This is due to a role of this motor in delivery of dynein to microtubule plus ends (175, 176) (Fig. 4). Dynein motors accumulate at the plus end to form a "dynein comet" (175, 184). Studies of *U. maydis* have shown that the apical dynein comets consist of ~55 dynein motors. Two mechanisms cooperate in the formation of the comet, namely binding of the dynein-associated complex dynactin to EB1-like proteins at microtubule plus ends and by a stochastic crowding effect (185). Dynein is released from the dynein comet and, while moving toward the subapical minus ends, can randomly pick up kinesin-3-delivered EEs (186) (Fig. 4). The large numbers of dyneins at plus ends increase the probability of such interaction. This ensures that EEs do not fall off the microtubule when they arrive at plus ends, but rather are transported back toward minus ends (185) (Fig. 4).

After dynein takes over, moving EEs still bind kinesin-3 (179, 186). Surprisingly, the amount of kinesin-3 on these organelles is ~4 times higher than that of dynein (186). This suggests that kinesin-3 activity is repressed upon dynein binding. While the exact mechanism underpinning this regulation is not known, the recent iden-

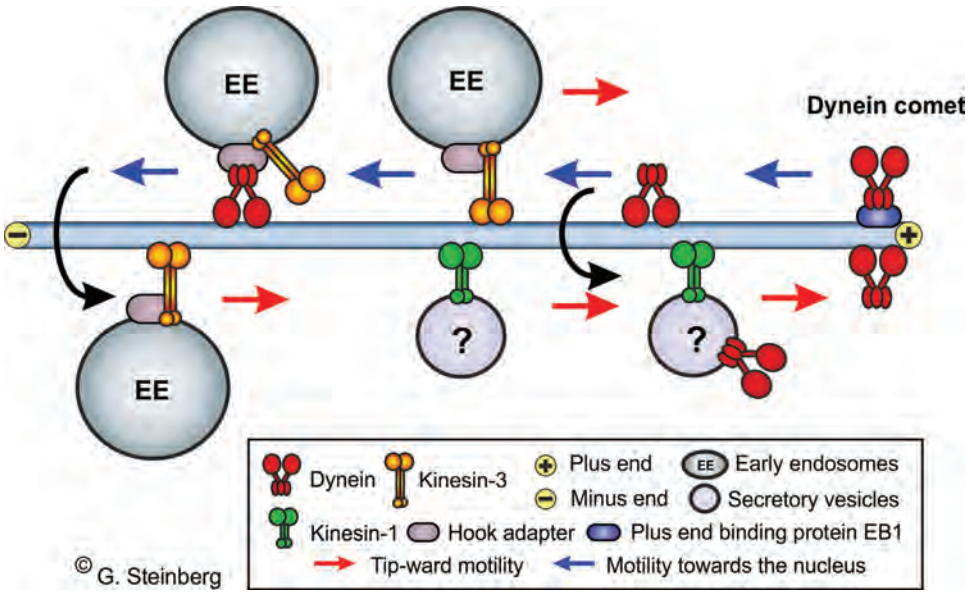


Figure 4 Diagram showing the cooperation of molecular motors in bidirectional EE motility. The illustration is based on results obtained from studies of *U. maydis*. See text for detailed description.

tification of an EE-located motor adapter complex provides the first insight into the complex interplay between kinesin-3 and dynein during bidirectional EE motility. The key protein in this EE adapter complex is a hook protein, which was identified in a genetic screen for mutants defective in EE motility in *A. nidulans* (187) and *U. maydis* (188). Hook forms a complex with two associated proteins, named FTS and FHIP (188), that help anchor the adapter complex to the EE membrane (189). Hook itself interacts with dynein (187, 188) and kinesin-3 (188) and is therefore a good candidate for controlling the activity or the attachment of both motors during bidirectional EE motility (15). This notion is supported by the finding that some kinesin-3 is released from EEs shortly before dynein binds (188). It is likely, however, that more proteins participate in this regulation, because for example, the dynactin subunit p25 is needed for dynein-dependent EE motility (190). However, EE motility may also be regulated by proteins that are not part of the transport machinery or the EE adapter complex. This is illustrated by the vezatin-like protein VezA in *A. nidulans* (191). Mutants in *vezA* show defects in dynein-hook interaction and, consequently, are impaired in retrograde EE motility. Surprisingly, VezA is not located on EEs, and the mechanism by which it controls interaction of dynein and EEs is not fully understood. Furthermore, the microtubule tracks themselves could participate in regulation of EE motility. It was shown in *N. crassa* and *A. nidulans* that kinesin-3 moves cargo preferentially along a subset of modified and less dynamic microtubules (165, 180). However, such selective transport of kinesin-3 was not found in the basidiomycete *U. maydis* (192).

Multiple Functions for Fungal EEs

The discovery of kinesin-3 as the motor for plus end-directed motility of fungal EEs (178) allowed the generation of mutants where EE motility was inhibited. This opened new avenues to investigate the role of these organelles in filamentous fungi. Over the past 10 years, intensive studies of *U. maydis* and *A. nidulans* have revealed multiple roles of EEs in fungal growth and virulence (Fig. 5).

The role of EEs in the endocytic pathway

EEs are the first compartment that sorts incoming material for recycling back to the plasma membrane or for degradation to the vacuole. It was shown that endocytic recycling supports tip growth of hyphae in *A. nidulans*, *A. oryzae*, *N. crassa*, and *A. gossypii* (37, 38, 152, 153, 156, 157). In addition, endocytic receptor recycling is essential during the early steps of pathogenicity in *U. maydis* (155). A role of EEs in recycling

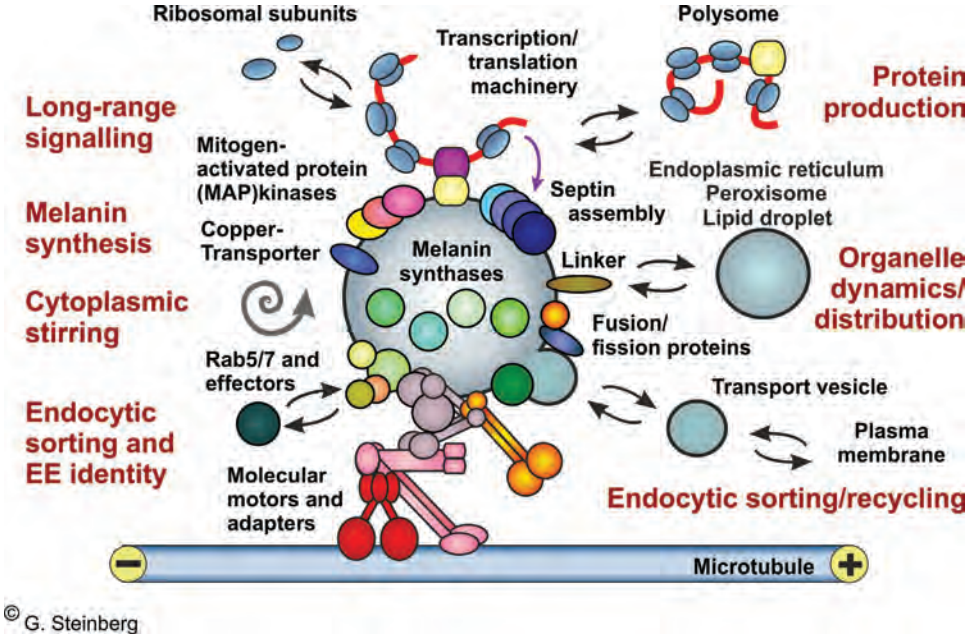


Figure 5 EEs as multifunctional platforms. Proteins that associate with the organelles are shown as colored symbols and described in black; functions are indicated in dark red. The diagram is based on work with *U. maydis*, *A. nidulans*, and *A. fumigatus*. See text for detailed description.

is also indicated by the fact that fungi contain the small GTPase Rab4 (155), which is involved in endocytic recycling at mammalian EEs (164). However, experimental evidence for such a role of Rab4 in filamentous fungi is missing. The second pathway from the EEs involves a maturation process, where Rab5 is replaced by Rab7, as shown in mammals (193), *A. nidulans* (194), and *U. maydis* (161). This maturation process from an EE to a late endosome and finally delivery to the vacuole involves EE motility (161, 194) and a complicated machinery of additional protein factors (75, 195). *A. nidulans* mutants defective in such factors (e.g., Vps33 or $PepA^{Pep12}$) (75) do not form normal colonies, which demonstrates that sorting to the vacuole is crucial for normal hyphal growth.

Hitchhiking of the protein translation machinery and organelles on EEs

Fungal EEs are constantly moving, and their motility is fueled by hydrolysis of ATP (15). The costs for the cell to maintain EE motility are best illustrated by a simple calculation, using parameters from *U. maydis*. Here, ~100 EEs are constantly moving (M. Schuster and G. Steinberg, unpublished results) at a rate of ~2 μm/s (186). Considering a step size of 8 nm (168, 169) and consumption of one ATP per step (196), these 100 EEs consume 90 million ATPs per hour. While EE motility is required to sort endocytosed material to the lysosome (see above), this high energy cost suggests additional functions of EE motility. Indeed, recent studies in *U. maydis* and *A. nidulans* have shown that other cellular cargos can "hitchhike" on moving EEs. The first report of such a mechanism demonstrated that the *U. maydis* mRNA-binding protein Rrm4 travels on EEs (197). Rrm4 links various mRNAs to a FYVE-domain-containing protein, Upa1, which itself is expected to bind to PIP3 lipid (198, 199). While it was initially suggested that this hitchhiking mechanism delivers mRNAs to the ends of the hyphal tip cell (200), a more recent report shows that entire polysomes (ribosomes and mRNA) bind and unbind to EEs, which distributes the protein translation machinery in the cell (161). Thus, transient hitchhiking supports the spatial organization of the translational machinery in the fungal cell.

Interestingly, it was reported that this mechanism is also used to relay organelles. In *U. maydis* (201) and *A. nidulans* (202), peroxisomes also transiently hitchhike on moving EEs. Moreover, lipid droplets and, to lesser extent, ER are distributed by moving EEs (201). Interestingly, when EE motility was blocked, organelles clustered at the hyphal tip (187, 201, 202). It was shown in *U. maydis* that this is due to a pole-ward drift of these organelles, probably powered by myosin 5-dependent delivery of secretory vesicles to the hyphal apex (203). Thus, hitchhiking on moving EEs maintains an even distribution of organelles and polysomes, which is vital for protein production and organelle function. It is worth noting that the process of hitchhiking is not restricted to filamentous fungi but may be a more general mechanism of cargo distribution in eukaryotic cells (204).

Local protein translation on moving EEs

Recent studies of *U. maydis* revealed a novel aspect of mRNA hitchhiking on EEs. It was shown that polysomes, while bound to moving EEs, are translationally active (161, 205). Initially, it was thought that this mechanism may ensure binding of ribosomes to EE-anchored mRNA for cellular distribution (161). However, recent results in *U. maydis* show that all core septin proteins (Cdc3, Cdc10, Cdc11, Cdc12) travel on EEs in an Rrm4-dependent way (206). This suggests that septin mRNA is traveling on EEs and subsequently being translated on the organelles to septin proteins, which assemble into a heteromeric septin building block. Thus, local translation on EEs may help to assemble and transport protein complexes in the hyphal cell.

Mixing the cytoplasm to enhance organelle interactions

EEs are constantly moving along microtubules (186). This raises the possibility that the EE trafficking stirs the fungal cytoplasm. Evidence for such a function was recently provided in *U. maydis*. Here, bidirectional EE motility increases the diffusion of peroxisomes and lipid droplets (203). This is most likely due to perturbation of the cytoplasm or direct collision between EEs and organelles. These unspecific interactions are further fostered by dynein-driven bending of microtubules (207, 208), which allow moving EEs to explore all regions of the cytoplasm within the hyphal cell tube. Mathematical modeling shows that these perturbations increase the local mobility of peroxisomes and lipid droplets, thus raising the chance of organelle-organelle interactions, which are essential for the cellular roles of both organelles (209). Interestingly, EEs in *A. nidulans* were shown to support the aggregation of proteins, which are formed in response to a heat shock (210). How EEs support protein aggregation is not known, but transient interactions between both were reported (210), suggesting that either hitchhiking (see above) or passive collisions support protein aggregate formation.

Participation of EEs in melanin biosynthesis

Melanin is a negatively charged hydrophobic polymer pigment. This polymer mechanically strengthens the fungal cell wall and protects microorganisms against microbicidal peptides, reactive oxygen species, and antifungal drugs. Consequently, melanin is associated with fungal virulence, in both human and plant pathogens (211–213). A genetic screen, designed to identify conidial pigmentation mutants in *A. fumigatus* (214), revealed a novel role for EEs in melanin biosynthesis. In one of the mutants, all melanin synthesis genes were unaffected, but a conserved sorting nexin, Mvp1, was mutated (215). This protein contains a PHOX domain, known to bind to lipids enriched in EEs (162). Subsequent studies led to the discovery that early enzymes of the melanin biosynthesis pathway (Alb1, Arp1, Arp2, Ayg1) are recruited to EEs. This, and the role of the sortin nexin Mvp1 in conidia pigmentation, strongly suggest that fungal melanin biosynthesis is initiated in the endosomal system, from where melanin intermediates are secreted and further processed by late biosynthetic enzymes (Abr1, Abr2) in the cell wall. Thus, EEs may govern aspects of secondary metabolism in filamentous fungi.

Endosomes in Long-Range Signaling

Mammalian neurons and elongate fungal hyphae share a common challenge in long-range signaling from the cell pole to the nucleus. In *U. maydis*, the nucleus is located ~50 μm behind the growing tip (216), and perception of external cues by tip-located receptors requires signaling over this distance. In neurons, such retrograde signaling is supported by moving EEs (217), and it was speculated that fungal EEs also take part in long-range transmission of signals (218). Evidence for this hypothesis comes from work with *U. maydis*. When the fungus enters into its plant host, it secretes effector proteins to suppress plant defense system activation (219–222). But how does the fungal nucleus know that effector genes need to be transcribed? It was shown that retrograde EE motility bridges between the invading hyphal tip and the subapical nucleus and that this motility is required to increase effector gene transcription and subsequent effector protein secretion (219). This supports the notion that EE motility transmits the signal for effector production from the hyphal tip to the subapical nucleus. Neither the plant cues that trigger effector transcription nor the detailed mechanism underpinning signal relay is known. However, the mitogen-activated protein kinase Crk1, described in the control of morphogenesis in *U. maydis* (223), is located on EEs and participates in regulating effector transcription (219). While our understanding of long-range signaling in fungi is still very fragmentary, these findings demonstrate a role for moving EEs in signal transduction.

Since the discovery of EEs in 2000 in *U. maydis* (160), our knowledge of the molecular machinery and cellular role of EE motility has increased significantly. It has emerged that EEs are motile multipurpose platforms that are involved in various essential aspects of fungal biology (Fig. 5). Initially recognized as sorting organelles, involved in recycling and degradation of endocytosed cargo, EEs have now been shown to spatially organize the hyphal cell. They enable long-range signaling and serve as platforms for local protein translation. Thus, motile EEs are pivotal to the fungal cell.

SEPTATION IN FUNGAL HYPHAE

Septum Formation

Fungal cells divide via the process of septation, whereby the localized synthesis of a cross-wall divides an existing cell into two distinct cells. In yeasts, dissolution of the septum physically separates the two cells. However, they remain attached in multicellular hyphae and often retain some degree of cytoplasmic continuity through septal pores. It is well established that the process of septum formation in fungal hyphae requires the presence of intact actin filaments, which assemble into a ring (i.e., the contractile actin ring [CAR]) (Fig. 6) that constricts in a process resembling cytokinesis in animal cells (224, 225). In yeasts, and presumably hyphae as well, the CAR guides deposition of the septal wall material (224, 226). Much of our understanding of the mechanisms that underlie assembly and constriction of the CAR is based on the functional characterization of gene products identified on the basis of their homology to yeast proteins with well-defined roles in septation (e.g., references 226–228). In general, the roles of these gene products appear to be largely conserved in filamentous fungi (224). Additional studies have provided insight into the regulation of septum formation in fungal hyphae, and in some cases they have yielded unexpected surprises (229). These surprises to a large extent reflect novel features of septum formation in multicellular hyphae compared to yeasts. This includes, for example, the precise spacing of septa so that the appropriate compartment size is maintained (230). Achieving a greater understanding of the mechanics and regulation of septum formation in fungal hyphae will go a long way toward revealing key functional features that underlie the unique organization of fungal hyphae.

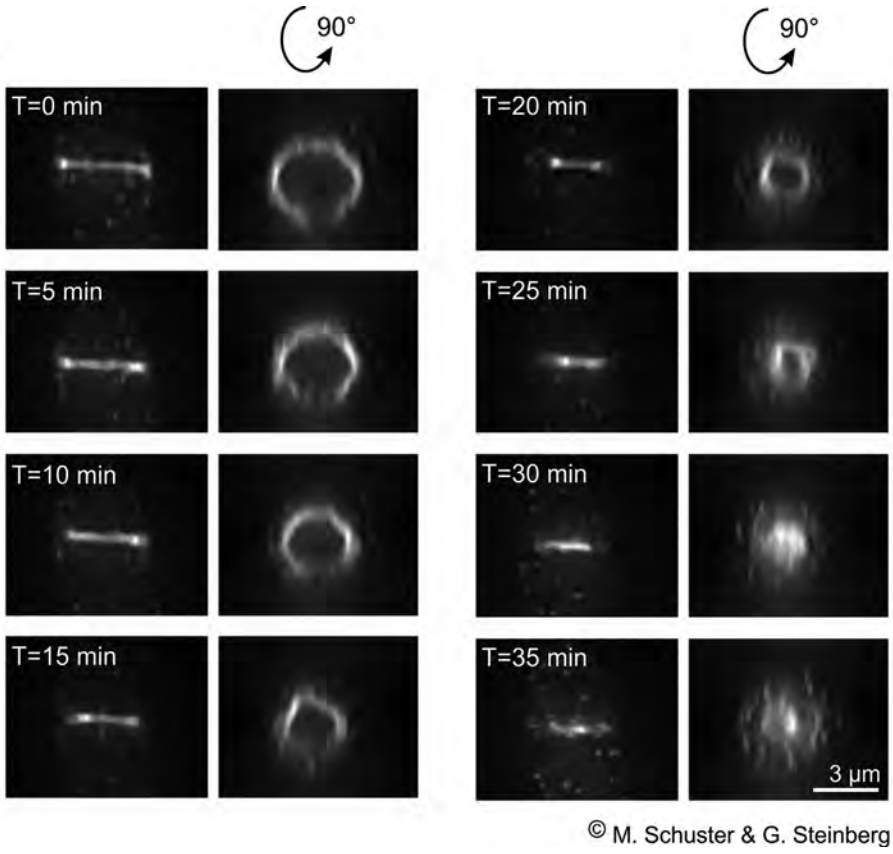


Figure 6 Time course of the contraction of the CAR during septum formation in the wheat pathogen *Z. tritici*. The side view of the three-dimensional image stack shows that the CAR is closing with time. Time in minutes is shown in the upper-left corners. The CAR was labeled using an F-actin-specific GFP-LifeAct probe.

The CAR and deposition of the septum

Like in animal cells, assembly and constriction of the CAR underlies septum formation in fungal hyphae (224, 225). The CAR initially appears at the incipient septation site as a tangle of actin filaments that is associated with myosin and tropomyosin. This structure subsequently consolidates into a thin "proto-CAR" that circumscribes the hypha. Constriction of the CAR coincides with ingrowth of the plasma membrane and the deposition of the new cell wall material that will become the septum. The CAR remains positioned at the advancing edge of the invaginating plasma membrane, which is also the active site of cell wall deposition that is mediated by the delivery of exocytic vesicles. During constriction, the appearance of actin patches that flank the CAR is consistent with localized endocytosis. Unlike cytokinesis in yeast cells, the CAR does not fully close, thereby retaining cytoplasmic continuity between adjacent hyphal cells through the resulting septal pore.

Other than tropomyosin and myosin, other components of the CAR include the anillin-like protein Bud4 (27, 224, 231), formin (224, 232), the actin-nucleating protein Bud6 (233), alpha-actinin (234, 235), paxillin (A. Virag and S. D. Harris, unpublished), and chitin synthase (224, 236). Notably, Bud4 and chitin synthase appear just prior to the initiation of constriction (224) (Fig. 7). Depending on the specific fungus, the absence of any of these components completely abolishes septum formation or results in the formation of abnormal septa. In general, formins, Bud6, and alpha-actinin are directly needed to assemble microfilaments into a CAR (232, 233, 235). The role of Bud4 is particularly intriguing, because Bud4 homologues in *S. cerevisiae* and *S. pombe* associate with septins (237, 238). In filamentous fungi such as *A. nidulans*, *A. fumigatus*, and *N. crassa*, septins also appear as rings at septation sites (e.g., references 239–241). In addition, fungal septins are subject to posttranslational modification such as phosphorylation (242), which likely plays a key role in

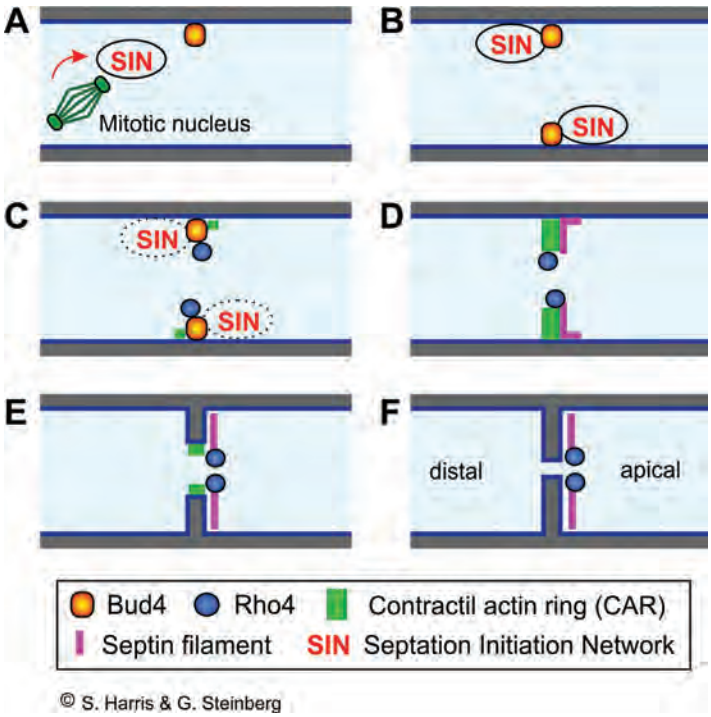


Figure 7 Model for septum formation. (**A**) A signal emanating from mitotic nuclei is relayed to the septation site via the septation initiation network (SIN). (**B**) Components needed for assembly of the contractile actin ring (CAR) (actin filaments, Bud4) are already associated with the septation site and operate in conjunction with the SIN to define the division plane. (**C**) Activation of the GTPase Rho4 at the septation site initiates organization of actin filaments into a CAR. (**D**) Constriction of the CAR is coincident with appearance of a septin ring. (**E**) Deposition of the septum is guided by the CAR. The septin ring disassembles once the final size of the septal pore is reached. (**F**) Several proteins, including calcineurin and Rho4, remain associated with the mature septal pore. The diagram was modified from Beck et al. (345).

determining their association with diverse partner proteins. Nevertheless, the relationship of septin localization and modifications to CARs and the timing of their appearance remain to be determined.

Studies using *A. nidulans* and *N. crassa* show that the monomeric GTPase Rho4 coordinates assembly of the CAR at septation sites (28, 243). Strikingly, Rho4 orthologues are conserved in filamentous fungi and *S. pombe*, but not in *S. cerevisiae* (which possesses a different GTPase known as Rho4). Activated Rho4 appears to recruit formins to the septation site (28), which is presumably an early step in the formation of the proto-CAR. It is interesting to note that weak homologues of *S. cerevisiae* Bud3 serve as guanine nucleotide exchange factors that activate Rho4 (27, 28). Accordingly, *rho4* and *bud3* mutants fail to recruit formins and thus do not assemble CARs or form septa. These results suggest that the Bud3-Bud4 module that provides a septin-associated spatial landmark for polarity establishment in budding yeast could conceivably have been co-opted from an ancestral function in directing CAR assembly at septation sites in filamentous fungi.

Regulation of septum formation in fungal hyphae

The formation of CARs at septation sites is subject to both spatial and temporal regulation (53). The existence of spatial regulation is supported by the uniform distribution of septa in fungal hyphae and their general exclusion from apical regions. Temporal regulation is likely based on the coordination of septum formation with mitosis at least in those filamentous fungi that exhibit parasynchronous nuclear division. Collectively, both forms of regulation presumably ensure that the compartment sizes in a hypha are appropriate for the extant growth conditions.

In *A. nidulans*, results from the use of mitotic inhibitors support the view that signals emanating from

dividing nuclei (e.g., the mitosis spindle or spindle poles) trigger the local formation of CARs (225). A promising source for such a signal is the septation initiation network (SIN), which coordinates CAR assembly and constriction with mitosis in *S. pombe* (244) (Fig. 7). Elements of the SIN pathway are conserved in filamentous fungi and are needed for CAR formation and septation (e.g., references 245, 246). However, they appear to function in a manner that is different from *S. pombe*, because for example, prior association with spindle poles is not required for SIN recruitment to septation sites (247). Although these observations implicate the SIN in the temporal regulation of CAR assembly or maturation, the specific nature of the signal remains to be determined. The timing of septum formation is also subject to regulation by DNA damage and other forms of genotoxic stress (229, 248). For example, low levels of DNA damage permit nuclear division but block septation in *A. nidulans* and *Fusarium graminearum*. In *A. nidulans*, this effect is mediated by inhibition of the same CDK that regulates mitosis (229).

Even though signals emanating from mitotic nuclei are presumably capable of triggering CAR assembly or maturation, it is also evident that not all mitoses are capable of activating formation of a septum. Otherwise, hyphal cells would in principle contain only a single nucleus, which is not the case for many filamentous fungi. Indeed, the regular spacing of septa and the relatively uniform number of septa per hyphal compartment in these fungi imply that septum formation is spatially regulated. That is, the activation of CAR assembly by a given mitosis event seemingly precludes adjacent dividing nuclei from also triggering septation. This could be simply due to titration of one or more key components needed for CAR assembly, but it might also reflect the active repression of septum formation at sites flanking a new CAR even if mitotic nuclei are nearby. How this might occur remains a puzzle, though it is tempting to speculate that cortical landmarks might specify "preseptation" sites that must be activated by an adjacent mitosis event to enable CAR formation or they are otherwise disassembled.

Communication and Differentiation of Hyphal Compartments

In basidiomycetes and ascomycetes, septa divide the hypha into compartments. These septa contain pores of 50 to 500 nm (249–252) that allow intra- and interhyphal translocation of metabolites, proteins, RNA, and even organelles. The rate of mass flow of cytoplasm in the mycelium of the basidiomycetes *Armillaria mellea* and *Serpula lacrimans* is in the range of 3 to 70 μm s^{-1} (253), while a rate of ~5 μm s^{-1} was observed in *N. crassa* (254). These rates underpin the potential for fast mixing of cytoplasm through the mycelium, implying that the mycelium of ascomycete and basidiomycete fungi is continuous like that of lower fungi. This paradigm is, however, changing. Studies have shown that fungal septa with growing hyphae can be closed, thus preventing cytoplasmic exchange between compartments (see "The Mechanism of Septal Pore Closure," below), which allows heterogeneity of hyphal cells within the fungal mycelium.

Hyphal heterogeneity

Zones within fungal mycelia show heterogeneous gene expression patterns and heterogeneity in growth and secretion (255–260; overview in reference 17). For instance, growth is mainly limited to the periphery of colonies of *Aspergillus niger* (255), while protein secretion occurs throughout the colony, with the exception of an intermediate zone, that is primed to sporulate under particular environmental conditions (260). There is differential gene expression across these zones (256, 259), and different proteins are secreted at the periphery and central zones of these colonies (259). Even hyphae within a given mycelial zone are heterogeneous with respect to cytosolic composition (17, 255, 257, 258, 261–265). For instance, part of the hyphae at the outer zone of *A. niger* colonies have high transcriptional and translational activity, which is accompanied by secretion of proteins that are involved in substrate degradation (255, 263–265).

Hyphae show highly polarized apical tip growth. They have at least four regions. The most apical region of hyphae (i.e., the first 1 to 5 μm) contains the Spitzenkörper, some mitochondria, and some smooth endoplasmic reticulum (266, 267). The tip region is also rich in actin filaments but sparse in ribosomes (262, 268). The second region is 2 to 4 times as large and contains mitochondria and some ER cisternae (104). The third region, which ends at the first septum, contains the complete collection of organelles. The fourth and following regions make up the second and following compartments. They contain all organelles, but their structure and abundance are often different from those in the third region. For instance, vacuoles are more abundant in subapical compartments, while 18S rRNA is less abundant, at least in the basidiomycete *Schizophyllum commune* (262).

Mechanisms of cytoplasmic flow

Cytoplasmic streaming depends on the architecture of the mycelium and is the result of turgor pressure

gradients, diffusion, and/or turbulence due to movement of organelles along the cytoskeleton. Hyphal fusion plays an important role in long-distance translocation of nutrients in *N. crassa* (269). This is evident from the finding that transport of nutrients is highly affected in a soft mutant (Δ*so*), which is a mutant strain that lacks the ability to undergo hyphal fusion. Even a 50% reduction in hyphal fusion frequency, as occurs in the Δ*prm-1* mutant, results in decreased nutrient translocation rates from the center toward the periphery of the colony (269).

Turgor pressure-mediated translocation has been proposed as a mechanism of cytoplasmic streaming in several fungi (253, 269–271). For instance, long-distance translocation of the nonmetabolizable glucose analogue 3-*O*-[^{14}C]methyl glucose through the mycelium of *Morchella esculenta* would depend on this mechanism. Translocation of 3-*O*-methyl glucose depends on the formation of sclerotia. A mutant unable to form these survival structures does not transport this glucose analogue through the mycelium (270). To explain these results, it was postulated that nutrients and solutes lower the water potential of the cytosol in the vegetative hyphae, resulting in water uptake from the medium. This uptake generates a higher turgor pressure in the hyphae. However, it is insufficiently high to create a flow through the mycelium. For this to occur, solutes are converted into high-molecular-weight polymers upon their arrival at the sclerotia or are released as exudate into the medium. These processes decrease the turgor within the sclerotia and thus act as a sink by attracting water (i.e., cytosol) from the vegetative hyphae. Addition of the metabolic inhibitor azide strongly reduces translocation of 3-O-methyl glucose, showing that metabolic activity is a prerequisite for turgor pressure gradient-mediated translocation to occur.

Mass transport driven by pressure gradients also dominates intrahyphal transport toward the hyphal tips of *N. crassa* (272). The pressure gradient required for this flow is very low (10^2 to 10^4 Pa cm^{-1}) compared with the 4 to 5 10^5 Pa turgor pressure within these hyphae (273). This low required value of the gradient is caused by the micrometer-range width of the hyphae. Pressure differences in micro-channels in the range of 10^2 to 10^3 Pa result in translocation rates of 50 to 500 μm s^{-1}, with mixing of the fluid dominated by diffusion (274). This velocity agrees with the maximal rate of bulk flow in *N. crassa* hyphae of 60 μm s^{-1}, although average flow rates are only ~5 μm s^{-1} (254). The intrahyphal osmotic gradients required for mass flow in *N. crassa* are likely to result from ion transport, as indicated by experiments using extracellular osmotic gradients. The high concentration of organelles in the cytosol of *N. crassa* hyphae impedes bulk flow of the cytoplasm, causing a so-called partial plug flow (272).

Cytoplasmic streaming in *N. crassa* occurs at ~5 μm s^{-1} (254). In contrast, the *N. crassa* kinesin motor Nkin transports its cargo at 2.1 to 3.8 μm s^{-1} (275). Thus, it is very unlikely that fast cytoplasmic streaming is a direct consequence of motor activity. Indeed, movement of nuclei toward the outer part of the colony was still observed in strains with mutations in microtubule-related motors (dynein and kinesin) and after treatment of wild-type strains with molecules disrupting the cytoskeleton (276). However, motor proteins move organelles in hyphal cells (overview in references 12, 167, 277, 278). Such motor-driven motility of organelles was recently shown to mix the cytoplasm and increase diffusion within the cytoplasm (203), which could support the exchange between adjacent hyphal cells. This notion is supported by findings in *A. niger*, in which depolymerization of actin and tubulin with cytochalasin A and nocodazole, respectively, reduced the rate of cytoplasmic streaming by about 25% (279). While cytoplasmic streaming was shown to support apical translocation of organelles (272, 280), it may also result in an uneven distribution of organelles, as was shown in *N. crassa* (281). Cytoplasm streams toward the apex in this fungus. The stream narrows in the septal pores, resulting in microfluidic eddies on the upstream side of the septum, where nuclei can be trapped. The presence of septa in hyphae thus creates subcellular domains within *N. crassa* hyphae.

It is currently not known if the mechanism underlying cytoplasmic streaming in *N. crassa* applies to all its hyphae or to other fungi. Most of the work on streaming in *N. crassa* has been done with so-called trunk hyphae. These hyphae are wider than the majority of hyphae in the mycelium. Such trunk hyphae have also been observed in other fungi such as *A. niger* (282), where they also represent the minority; cytoplasmic streaming in *A. niger* hyphae has a rate 2,000 times lower than that observed in *N. crassa*, while its growth rate is about 10-fold lower (279). The cytoplasm of fungal cells is able to contract, which was shown in *A. niger*, *N. crassa*, and *Trichoderma atroviride* (283, 284). Thus, motor-driven contractions of the cytoskeleton may contribute to cytoplasmic streaming in filamentous fungi.

Selective transport across closed septa

Fungi have the ability to close their septa, which limits exchange between hyphal compartments (see "Communication and Differentiation of Hyphal Compart-

ments," above). In *A. niger* it was shown that the newly formed compartments are in exchange with neighboring compartments, while older parts of the mycelium are closed off by plugging of the septal pore, with ~90% of the 4th to 8th subapical septa closed, followed by complete sealing of the 9th and 10th septa (279). Closure of these septa is mainly caused by a peroxisome-like organelle, the Woronin body (see "Closing Pores by Woronin Bodies," below). Due to the closure of old septa in the center of the colony, transport of nutrients through the cytoplasm cannot occur. Indeed, measurement of a fluorescent glucose analogue provided no evidence for glucose transport from the periphery to the colony center (282). Surprisingly, however, glucose was found to be transported from the center to the periphery of the mycelium. Because the fluorescent glucose analogue accumulated in the cross-walls of the septa, it was concluded that glucose is efficiently transported across the plasma membrane lining the septal cross-walls (282). This could be mediated by transporters that allow the passive passage of sugars down a concentration gradient. Indeed, a hexose transporter, *mstE*, localizes to septa in *A. nidulans* (285). Such a septal pore-independent and selective transport mechanism would operate independently of septal pore plugging (Fig. 8). Indeed, transport of glucose between different zones of the colony of *A. niger* was not increased in mutants that were devoid of Woronin bodies (282).

The Mechanism of Septal Pore Closure

Filamentous fungi grow by apical tip growth of their hyphae. In ascomycete and basidiomycete fungi and a few subgroups of the zygomycetes, septa partition the hypha into subcompartments. Communication between these compartments is mediated by septal pores, which range in size from 25 nm in *C. albicans* (286) to 360 nm in *N. crassa* (287). The width of the septal pore allows exchange of cytoplasm, including small molecules, ribosomes, and proteins, but also entire organelles such as mitochondria and nuclei (252, 254, 288). However, it is essential for the hyphal cell to keep this exchange under tight control and thus be able to close the septal pore upon stress or cell injury or during particular developmental stages and differentiation. To this end, fungi have developed various ways to plug the septal pore (289). In most cases, insight into the process of pore closure is based on ultrastructural studies, which show closure by organelles, small crystalline bodies, or electron-dense proteinaceous material (289). However, recent research has begun to shed light on the molecular mechanisms underpinning pore closure in filamentous fungi.

Closing pores by proteinaceous material

Plugging of the septal pore by a deposit occurs independently of hyphal injury and is based on *de novo* deposition of proteinaceous material (289). Basidiomycete fungi contain a complex septal pore, the dolipore (252), which was shown in *S. commune* to be plugged by electron-dense proteinaceous material in response to environmental stress (e.g., osmotic shock) or hyphal wounding (290, 291). Such deposition appears to be the only mechanism for pore plugging in basidiomycetes (subphyllum Agaricomycotina). Not much is known about the nature of this electron-dense material. However, it has been suggested that the septal pore cap

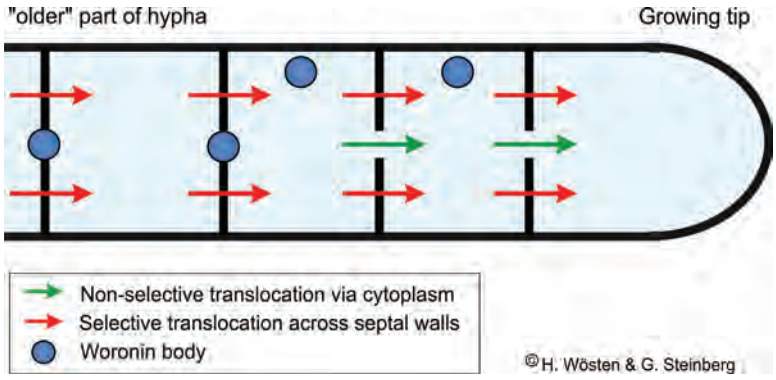


Figure 8 Model for tip-ward translocation in *A. niger*. Newly formed hyphal compartments are in cytoplasmic contact with neighboring cells. The Woronin body is not plugging the septal pore, and cytoplasmic streaming, as well as diffusion through the septal pore, is possible (green arrow). Older septa are plugged by Woronin bodies, which prevents exchange of cytoplasm. However, selective transport of molecules such as glucose toward the growth region is still possible (red arrows). This may involve septum-associated transporters.

(i.e., parenthesomes; Fig. 9) (252) of the basidiomycete dolipore provides a reservoir from which plugging proteins are released (289). The septal pore cap is derived from the endoplasmic reticulum and flanks both sides of the pore channel. Septal pore caps are usually perforated and were suggested to act as sieves that limit passage of organelles through the dolipore (292); passage of nuclei through dolipores requires its degradation and conversion to a simple pore (293). Support for an additional role of the septal pore cap in pore plugging comes from the recent identification of protein SPC18 in *Rhizoctonia solani* (294). SPC18 localizes to septal pore caps but was also found in the pore channels, suggesting that the septal pore cap provides the material for plugging the dolipore. This notion is further supported by a mutant in a septal pore cap protein SPC33 in *S. commune* (295). This mutant lacks septal pore caps and is unable to close the dolipore channel upon wounding. Interestingly, this results in defects in growth and fruiting body formation, indicating that the ability to close the septal pore is a prerequisite for differentiation and development in basidiomycetes.

In older parts of hyphae in the ascomycete fungus *N. crassa*, septal pores contain accumulations of electron-dense material (287), suggesting that protein deposition limits flow between hyphal compartments. Work in *N. crassa*, *Sordaria macrospora*, and *Aspergillus oryzae* revealed a role for the SOFT/PRO40/SO1 protein (hereafter named SO) in plugging septal pores (296–298). SO is a cytoplasmic protein that, upon cell injury or environmental stress, relocates to the septal pore. Mutants lacking SO show slower plugging after cell injury (297) or are significantly impaired in septal closure (298). This suggests that deposition of SO proteins participates in sealing septa. Interestingly, only the C-terminal half of SO is involved in plugging septal pores in *A. orzyae*, whereas the N-terminal half appears to serve a septum-independent role in cell-cell fusion (299). This functional diversity may explain the broad phenotypic variation in SO mutants, which show defects in cell-cell fusion, aerial hyphae formation, and conidiation (298, 300).

Whether SO protein represents the electron-dense material in septal pores of ascomycetes (e.g., references

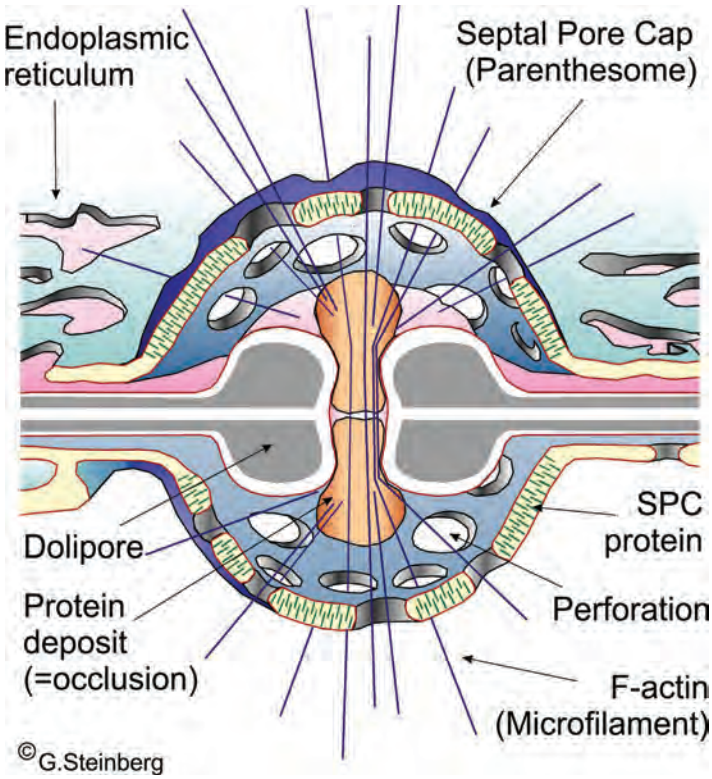


Figure 9 Schematic drawing of a dolipore in the basidiomycete *Polyporus biennis*. The image was redrawn from a reconstruction of electron micrographs, first published in reference 346.

287, 301) is not known. In *A. nidulans*, the NIMA kinase locates at the septum and the septal pore is open (302). Upon entry into mitosis, NIMA leaves the septum to control early steps in mitosis (303). Upon this relocation of NIMA, the septal pore closes, and this isolates the mitotic cell from neighboring interphase cells (302). Thus, NIMA appears to orchestrate septal pore opening during interphase. Surprisingly, this NIMA-controlled plugging occurs independently of SO and of Woronin bodies (see below), suggesting that additional mechanisms exist which close a septal pore. Indeed, the recent identification of 17 additional septal pore-associated (SPA) proteins (304) suggests that various proteins cooperate in pore closure. A subset of these SPA proteins localize to the lumen of the septal pore channel (SPA type II: SPA3, SPA5, SPA6). Thus, these proteins may participate in controlling communication and exchange between hyphal compartments. Such a role in pore plugging is further supported by biochemical data that show that recombinant SPA5 self-assembles into gel-like aggregates (304). However, the precise cellular role of most SPA proteins needs to be elucidated.

Closing pores by Woronin bodies (WBs)

Filamentous ascomycetes (subphylum Pezizomycotina) and imperfect fungi (Deuteromycota) have developed an additional mechanism for septal pore plugging. This is based on a fungus-specific organelle, the WB. WBs were named by A. H. Reginald Buller (305) after the Russian botanist Mikhail Strepanovich Woronin, who first described *kleine Körnchen* (small granules) adjacent to septa in *Ascobolus pulcherrimus* (306). Woronin's observations were confirmed in various fungi, where these highly refractive particles were found to move within the cytoplasm (see reference 305 for references). Subsequent electron microscopy studies revealed that WBs are spherical membrane-bound organelles of 100 to 750 nm in diameter (289). Fungal cells contain usually 3 to 6 WBs adjacent to their septa (305, 307), but a large number of additional WBs are found in the cytoplasm of *A. nidulans* and *A. fumigatus* (302, 308, 309). Septum-associated WBs are nonmobile and anchored at the septal pore (301, 308, 310). In *Neurospora* and *Sordaria*, WBs are larger and are anchored at the cell periphery, which is thought to be an adaptation to the prominent and rapid cytoplasmic streaming seen in these fungi (311).

The main purpose of WB-based septal pore closure is to prevent catastrophic loss of cytoplasm after hyphal injury (289). The first insight into such a function in septal pore plugging was provided by ultrastructural studies that showed WBs sealing septa in wounded hyphae (312–314). A breakthrough came with the identification of the protein Hex1 in WBs of *N. crassa* (315, 316), which was subsequently reported in *M. grisea* (317), *Trichoderma reesei* (318), *A. oryzae* (301), and *A. fumigatus* (308). Hex1 is the main component of the crystalline matrix of WBs (319, 320), and deletion of *hex* genes results in the absence of WBs (301, 308, 315, 316). Strikingly, these null mutants are impaired in sealing their septa after injury, which strongly indicates a role of WBs in septal pore plugging. However, WBs also seal 5 to 13% of septal pores in unwounded hyphae (321). This suggests that WBs have additional roles in restricting flow through septal pores in healthy hyphae, and this may generate heterogeneity within the mycelium (261).

Ultrastructural studies of *F. oxysporum* suggested that WBs originate from microbodies (i.e., peroxisomes and glyoxysomes; reference 322). This conclusion gained support by the finding that antibodies against the peroxisomal targeting signal recognize WBs (323). Hex1 from *N. crassa* contains a peroxisomal targeting signal, and live-cell imaging confirmed that Hex1 crystals are formed at peroxisomes (324), where a Woronin sorting complex (WSC) supports assembly of Hex1 and formation of WBs, as well as cortical anchorage of the organelle (325). A role for the WSC in WB formation was confirmed in *A. fumigatus*, although WSC was not involved in anchorage of WBs near the septum (326). Finally, it was shown that mutants deleted in peroxisomal *pex*-genes (*pex6*, *pex14*, *pex11*), required for proliferation or biogenesis of peroxisomes in *M. grisea*, *N. crassa*, and *A. oryzae*, lack WBs (327–329). Taken together, these studies provide compelling evidence for the conclusion that WBs derive from peroxisomes.

It was recognized early that WBs, once in position, are stationary and even resist fast cytoplasmic streaming (301, 305, 308, 324, 325). Interestingly, when WBs were misplaced by an optical laser trap in *N. haematococca*, they snapped back to their original position at the septum (330). This simple experiment strongly suggests the existence of a physical tether that holds WBs in position. Indeed, ultrastructural studies suggested a fibrous connection between WBs and the septal pore (309). Again, it was work with *N. crassa* that moved the field forward and revealed the molecular nature of this tether. A genetic screen for mutants defective in WB segregation identified the *leashin* (*lah*) locus, which is involved in WB tethering (331). This locus encodes two proteins, LAH-1 and LAH-2. While LAH-1 tethers WBs to the cell cortex, LAH-2 is located at the hyphal apex and the septal pore, where it performs unknown

functions. In most other ascomycetes, only LAH-2 homologues, consisting of 6,000 to 7,500 amino acids, are found that have been shown to tether WBs to the septum. In *A. oryzae* and *A. fumigatus* mutants that lack Lah proteins, WBs are no longer tethered to the septum and hyphae fail to plug their septal pores after wounding (326, 332). Most strikingly, a shorter version of Lah reduces the distance of WBs to the septal pore (from 99 to 50 nm) (332), which adds further evidence for a role of Lah in tethering WBs to septal pores. The molecular details of how Lah proteins tether the WB proteins may differ between fungi, because Lah was shown to interact with WSC in *N. crassa* (331), whereas Lah was reported to bind directly to HexA in *A. fumigatus* (326). How the cytoplasmic tether interacts with the WB core protein HexA is not understood.

The mechanism by which WBs move into the septal pore during plugging remains elusive. A tempting hypothesis is that hyphal injury results in high pressure in the adjacent cells, and bulk flow of cytoplasm moves WBs into the pore channel (310, 313). Indeed, sealing of septal pores takes several seconds, and hyphal cells often show some cytoplasmic bleeding before the pore is plugged (261, 297). However, such a passive mechanism does not explain why only one WB seals the septal pore while the others remain unaffected (307). Alternatively, it was suggested that an active, maybe cytoskeleton-dependent or contractile mechanism could draw WBs toward the septum (307, 310). The nature of such a mechanism is not known, but it may involve the tether protein Lah. Indeed, Lah shares short sequence motifs with the muscle protein titin (331, 332), which were shown to respond to changes in intracellular calcium, thereby changing the elasticity of the protein (333). Hyphal wounding increases calcium levels at the septal pore (304), which supports the idea that Lah could act as an elastic molecular spring that drags WBs into the septal pore.

In *A. nidulans* and *A. oryzae* the vast majority of the WBs are not associated with the septum (302, 308), but rather move rapidly along the cytoskeleton at a speed of ~1.4 μm/s (302). Similar behavior was described by early light microscopists (305 and references therein), suggesting that WB motility is a common feature. Currently, neither the molecular machinery for this motility nor the biological role of these moving WBs is understood. One possibility is that immature WBs are motile until they settle at the septum. Alternatively, WBs may have additional and septum-independent functions in the fungal cell. Assessing this question is further complicated by the fact that contradictory conclusions were drawn from *hex1* mutants in various fungi. While Hex1, and therefore WBs, in *A. oryzae* was found to be essential for conidiaton (301), *N. crassa* mutants deleted in *HEX-1* either showed no defect in conidiation (315) or exhibited poor conidiation (316). Moreover, in the plant pathogen *M. grisea*, the Hex1 homologue MVP1 was reported to be dispensable for virulence (317), whereas another study concluded that Hex1 in this fungus is highly important for its full virulence (334). The reason for such phenotypic variation is currently not known.

Consolidation

The final step after plugging the pore by protein deposit and/or WBs is the development of a permanent seal of the pore. This process, called consolidation, is found in asco- and basidiomycetes (289, 335). It involves synthesis and deposition of electron-lucent cell wall material at the septum and occurs within 30 min to 3 h after hyphal wounding (290, 307, 314). Cell wall formation requires delivery of secretory vesicles, which carry cell wall synthases along the cytoskeleton (135, 136, 336, 337). Indeed, large numbers of vesicles (314), as well as actin filaments and associated myosin-5 motors, concentrate at the septal pore of injured hyphae (304). Thus, consolidation results in permanent sealing, which follows the rapid emergency plugging of septal pores in response to hyphal cell injury.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this article, we have summarized our current knowledge of important processes underlying hyphal growth in filamentous fungi. We have shown that hyphal morphogenesis and septum formation are distinct morphogenetic processes that rely on the cytoskeleton and vesicle trafficking machinery to direct localized cell wall deposition to a precise location. Vesicle trafficking also characterizes the conventional secretory pathway in fungi. We have summarized our understanding of the processing of secretory cargo across the Golgi, coat-independent secretory vesicle budding from the trans-Golgi network, and the composition of the Spitzenkörper. Recent identification of nonclassical pathways and the role of fungal-specific myosin-chitin synthases in exocytosis add to the complexity of the secretory pathway in filamentous fungi. In addition, it has emerged that EEs are not only involved in the endocytic pathway; they are also crucial for the spatial organization of the hyphal cell and perform specialized roles in long-range signaling, melanin biosynthesis, septin filament assembly and organizing protein translation in the cell. Finally, we

have summarized our current knowledge of the mechanism of septum plugging and its role in controlling communication between hyphal cells.

While our understanding of various proteins and structures involved in these critical processes is growing rapidly, little is known about the regulatory mechanisms that orchestrate them. Current research is focused on a few model fungi; this limits comparative studies. However, comparative genomics among fungal species belonging to different taxa will allow identification of conserved key core determinants of hyphal tip growth. Furthermore, establishing molecular tools for cytological studies in nonmodel fungi, such as the wheat pathogen *Z. tritici* (166, 338–343) or the grass pathogen species *Ustilago bromivora* and *Brachypodium* spp. (344), will enable more detailed comparison of the cytology of hyphal growth and fungal pathogenicity.

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The Fungal Cell Wall: Structure, Biosynthesis, and Function

12

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INTRODUCTION

Fungal cell walls are dynamic structures that are essential for cell viability, morphogenesis, and pathogenesis. The wall is much more than the outer layer of the fungus; it is also a dynamic organelle whose composition greatly influences the ecology of the fungus and whose composition is highly regulated in response to environmental conditions and imposed stresses. A measure of the importance of the cell wall can be appreciated by the fact that approximately one-fifth of the yeast genome is devoted to the biosynthesis of the cell wall (1, 2). Of these approximately 1,200 *Saccharomyces cerevisiae* genes (2), some are concerned with the assembly of the basic components, others provide substrates for wall materials, and many regulate the assembly process and couple this to environmental challenges. They include genes that encode carbohydrate active enzymes (which can be found in the CAZy database [http://www.cazy.org]) (3) and include multigene families of chitin and glucan synthases as well as remodeling enzymes such as the glycohydrolases (glucanases, chitinases) and transglycosidases. Many of the building blocks of the cell wall are conserved in different fungal species (4), while other components of the wall are species-specific, and there is perhaps no part of the cell that exhibits more phenotypic diversity and plasticity than the cell wall.

Walls are built to be both malleable and mechanically robust. The high total solute concentration inside fungal cells results in the osmotic uptake of water and the pressing of the cell membrane onto the wall. The resulting turgor pressure has been estimated to be between 0.2 and 10 MPa—equivalent to 2 to 20 times atmospheric pressure (5). The melanized cell walls of the appressoria of some plant pathogens such as *Magnaporthe oryzae* can withhold an internal turgor of up to 20 MPa. These cell walls are the most robust of all walls found in nature. Turgor pressure generates the force that enables hyphae to exert mechanical force on the substrates they are penetrating (5, 6). Hyphal forces of between 0.01 and 0.1 μN/μm (5) can be exerted at the hyphal tips which are sufficient to enable most vegetative hyphae to penetrate a stiff 8% (wt/vol) agar. Wall-less cells are invariably spherical, so the cell wall of fungi also determines their complex shapes and changes in cell shape underpinning, morphogenesis, and cellular differentiation. Because the cell wall is also the natural interface between the fungus and its environment, it goes a long way to defining the ecology of fungi by influencing their interactions with substrates and other organisms.

For fungal pathogens of humans, the wall induces innate and adaptive immune responses, and the design of the cell wall sometimes incorporates immune decoys and shields (7). Similarly, for plant pathogenic fungi the cell wall is detected by receptors in the plant cell that induce local and systemic defense responses (8). The cell wall provides a valuable source of most diagnostic antigens that are used to detect human fungal infections, and it represents a rich source of unique targets for chemotherapeutic treatment of pathogens. Therefore, fungi are in no small measure defined by, and live through, the interface of their cell walls. Recent progress has been considerable, yet some of the most important and elusive questions in fungal cell biology relate to basic aspects of fungal cell wall biosynthesis and function. This review focuses on the biosynthesis and functions of fungal cell walls with an emphasis on model pathogenic species where the most detailed information is often available.

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COMPOSITION AND STRUCTURE

Structural Organization and Cell Wall Layers

Fundamentally, fungal walls are all engineered in a similar way. The wall structure directly affects wall function and interactions with the environment including immune recognition by plants and animals. Fibrous and gel-like carbohydrate polymers form a tensile and robust core scaffold to which a variety of proteins and other superficial components are added that together make strong, but flexible, and chemically diverse cell walls. Most cell walls are layered, with the innermost layer comprising a relatively conserved structural skeletal layer and the outer layers more heterogeneous and tailored to the physiology of particular fungi. In most fungal species the inner cell wall consists of a core of covalently attached branched β-(1,3) glucan with 3 to 4% interchain and chitin (9, 10). β-(1,3) Glucan and chitin form intrachain hydrogen bonds and can assemble into fibrous microfibrils that form a basket-like scaffold around the cell. This exoskeleton represents the load-bearing, structural component of the wall that resists the substantial internal hydrostatic pressure exerted on the wall by the cytoplasm and membrane. This branched β-(1,3):β-(1,6) glucan is bound to proteins and/or other polysaccharides, whose composition may vary with the fungal species (Fig. 1). However, yeast cells have bud scars that tend to have fewer outer cell wall layers covering them and therefore have exposed inner wall chitin and β-(1,3) glucan (11). The inner walls of many fungal spores and so-called black yeasts contain complex amorphous polymerized phenolic compounds called melanins, which also add protection—particularly from oxidants and some exoenzymes.

The outer layers of fungi vary much more than the inner skeletal layer (Fig. 1). Yeasts such as *Candida* and *Saccharomyces* species and the human pathogen *Pneumocystis jiroveci* have an outer cell wall comprising highly mannosylated glycoproteins that covers the inner wall. In the yeast cells of some polymorphic fungi such as the human pathogen *Histoplasma capsulatum*, the outer wall has a layer of α-(1,3) glucan that prevents dectin-1-mediated immune recognition of the underlying β-(1,3) glucan by immune cells (12).

α-(1,3) Glucan plays a prominent role in the organization of the cell wall of many human pathogens but is absent from the *Candida* and *Saccharomyces* cell walls. In *Aspergillus*, an α-(1,3) glucan along with some other amorphous polysaccharides is represented in the alkali-soluble cell wall fraction (13). The basidiomycetous yeast *Cryptococcus* has a cell wall that is enveloped by a gelatinous capsule composed of glucuronoxylomannan (GXM) and galactoxylomannan (Fig. 1) and that is anchored to the main wall via α-(1,3) glucan (14). The GXM (~90% of the mass of the capsule) is composed of α-(1,3)-linked mannan with glucuronic acid, xylose, and *O*-acetyl branches. The galactoxylomannan is composed of an α-(1,6) galactomannan (GM) backbone with GM side chains substituted with variable numbers of xylose residues (Fig. 1). The synthesis of the *Cryptococcus neoformans* capsule polysaccharides remains incompletely understood, and few transglycosidases involved in this process have been characterized (15, 16). Interestingly, it has been shown that capsule polysaccharides are synthesized intracellularly and secreted via exocytosis through the cell wall (17).

The conidial spores and aerial hyphae of mold are often covered by highly hydrophobic proteins called hydrophobins that form rodlets that protect the spores from enzymes, oxidants, and foraging phagocytes (18). For example, the conidial rodlet protein RodA of *Aspergillus fumigatus* prevents alveolar macrophages from inducing an immune response, which is delayed until this layer cracks upon spore swelling and germination (Fig. 1) (19, 20). However, once the integrity of the rodlet layer of these spores is breached, then the underlying galactosaminoglycan (GAG) and GM and β-(1,3) glucan layers can be recognized by alveolar macrophages, enabling the innate immune response to be initiated (21).

The individual components of the cell wall are covalently cross-linked to one another. In *A. fumigatus*, the branched β-(1,3)(1,6) glucan is covalently bound to chitin, a linear β-(1,3)(1,4) glucan with a [3Glcβ1-4Glcβ1] repeating unit and a branched galactomannan composed of a linear α-mannan with a repeating mannose oligosaccharide unit [6Manα1-2Manα1-2Manα1-2Manα] and short chains of β-(1,5) galactofuranose residues (22). In *Candida albicans*, β-(1,3) glucan is bound to chitin and β-(1,6) glucan, a polymer that is absent from *A. fumigatus*. In *C. albicans*, β-(1,6) glucan plays an essential role in the structural organization of the cell wall (23) interconnecting β-(1,3) glucan and chitin (Fig. 1). In *C. neoformans* and most other fungi, the covalent linkages between glucans and the other polymers have not been investigated.

In *C. albicans* and *S. cerevisiae*, β-(1,6) glucan acts as a linker molecule binding cell wall proteins (CWPs) to the β-(1,3) glucan-chitin skeleton via a glycosylphosphatidyl inositol (GPI) remnant (24). In yeast cells of these species, the CWPs represent 30 to 50% of the dry mass of the wall, of which only 10 to 20% is polypeptide. A few proteins with internal repeats (Pir)

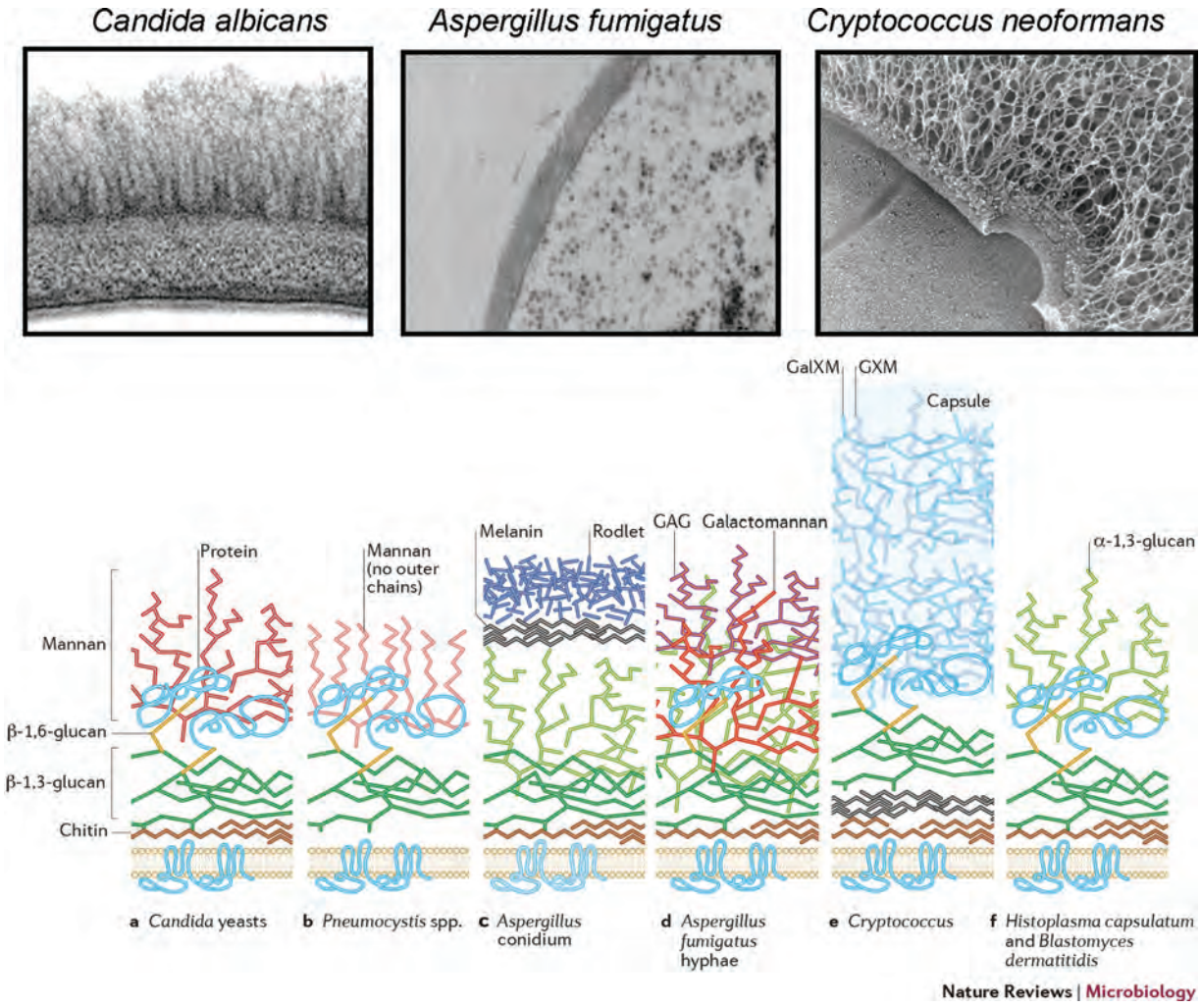


Figure 1 Structural organization of the cell walls of fungal pathogens. The upper panels show transmission electron micrograph sections of the cell walls, revealing mannoprotein fibrils in the outer walls of *C. albicans*, the fibril-free cell wall of an *A. fumigatus* hypha, and the elaborate capsule of *C. neoformans*. The cartoons (below) show the major components of the wall and current hypotheses about their interconnections. Most fungi have a common alkali-insoluble core of branched β-(1,3) glucan, β-(1,6) glucan, and chitin but differ substantially in the components that are attached to this. In *C. albicans*, the outer wall is heavily enriched with highly mannosylated proteins that are mostly attached via glycosylphosphatidylinositol remnants to β-(1,6) glucan and to the β-(1,3) glucan-chitin core. In *A. fumigatus*, typical of many filamentous fungi, mannan chains are of lower molecular weight and are modified with β-(1,5) galactofuran. These mannans are not components of glycoproteins but are attached directly to the cell wall core. The cell wall core polysaccharides of *A. fumigatus* are β-(1,3)-β-(1,4) glucans and are attached to an outer layer of alkali-soluble linear α-(1,3)(1,4) glucan. Conidial walls of *Aspergillus* have an outer hydrophobin rodlet layer of highly hydrophobic portions (hydrophobins) and a melanin layer; hyphae of *Aspergillus* have α-(1,3) glucan GM, and galactosaminoglycan (GAG) in the outer cell wall and limited glycosylated proteins. In *C. neoformans*, an outer capsule is composed of glucuronoxylomannan (GXM) and lesser amounts of galactoxylomannan (GalXM). The capsule is attached to α-(1,3) glucan in the underlying wall, although peptides or other glycans may also be required for anchoring the capsule to the cell wall. The inner wall has a β-(1,3) glucan-β-(1,6) glucan-chitin core, but most of the chitin is deacetylated to chitosan, and some of the chitosan/chitin may be located further from the membrane. *C. neoformans* also has a layer of melanin whose precise location is not known, but it may be incorporated into several cell wall polysaccharides and may assemble close to the chitin/chitosan layer. *Pneumocystis* cell walls may lack chitin and the outer chain *N*-mannans but retain core *N*-mannan and *O*-mannan modified proteins (56). Hyphae of *H. capsulatum* and *Blastomyces dermatitidis* have an outer cell wall layer of α-(1,3) glucan that prevents efficient immune recognition of β-(1,3) glucan in the inner cell wall. (From reference 7, with permission.)

can be attached directly to β-(1,3) glucan via an alkali-sensitive *O*-linkage via a mannose side chain (24). In *A. fumigatus*, the cell wall has a much reduced glycol protein content, and galactosylated mannoproteins are not cell wall-associated and are secreted proteins in transit in the cell wall.

This general arrangement places the structural elements of the cell wall close to the membrane to provide mechanical support, and places the gel-like or hydrophobic polymers to the outside where they can protect the load-bearing elements from degradative enzymes in the environment or act as adhesins to anchor the cell to substrata. A major unresolved issue is how the cell wall polysaccharides and membrane proteins are bound together to guide the cell wall morphogenesis.

Biofilms

Some of the external gel-like polymers of the outer surface of fungi are extremely well organized, such as in the capsule of *C. neoformans*, while other fungi, such as some *Candida* species, form biofilms growing on solid surfaces, where the extracellular matrix has a more loose structure composed of glucans, chitin, nucleic acids, and other polymers (25, 26). On top of the *C. neoformans* capsule there may also be a superficial biofilm that contains GXM as well as polysaccharides that differ from those found in the capsule, with significant amounts of glucose and fucose (27). Biofilms of *Pneumocystis carinii* and *C. albicans* are rich in β-(1,3) glucans and DNA (28–30), while *Candida tropicalis* has a biofilm matrix rich in GlcNAc (31). In *C. albicans*, growth of the fungus in a biofilm community results in cell wall architectural changes. In *A. fumigatus*, the extracellular matrix plays an essential role in the organization of the colony by gluing together mycelial threads and is composed of 25% polysaccharides and 70% monosaccharides with some hydrophobic proteins and melanin. The extracellular matrix of *A. fumigatus* contains α-(1,3) glucan, GM, and GAG, like the outer cell wall layer (32), and in contrast to other species, lacks β-(1,3) glucan and chitin. In human pathogens this biofilm material has been implicated in blocking recognition and immune capture by phagocytic cells.

GENETICS, ENZYMOLOGY, AND BIOSYNTHESIS

Biosynthesis of the Polysaccharides

Polysaccharides such as chitin and glucan are synthesized at the plasma membrane (PM) by transmembrane enzymatic complexes that are targeted to the PM in an inactive form via secretory vesicles and then activated after insertion into the PM (see below). This is in contrast to mannans and other glycoconjugates that are synthesized in the endoplasmic reticulum and Golgi, where they may be conjugated to cell wall proteins, and then brought to the cell wall by the classical secretory route via secretory vesicles. All synthases use nucleotide diphosphate-sugars as substrates, so enzymes of the metabolic pathways responsible for the synthesis of nucleotide sugars are essential for the construction of the cell wall and are rate-limiting.

Core Polysaccharides: Chitin and β-(1,3) Glucan

The major synthases that make chitin and glucans reside in the PM and use UDP-sugars as the substrate for the formation of the nascent polysaccharide that is extruded into the cell wall (Fig. 2A). In the cell wall, polysaccharides can then hydrogen-bond together or be cross-linked or branched by enzymes that reside in the cell wall (Fig. 2B,C). The pathway of cell wall synthesis therefore comprises biosynthetic reactions that take place inside the cell in the Golgi, at the PM, and in the cell wall itself.

UDP-*N*-acetylglucosamine is the substrate for chitin synthesis. Chitin is composed of linear chains of β-(1,4) *N*-acetylglucosamine and represents the most ancestral structural polysaccharide in the fungal cell wall. Families of chitin synthases responsible for the synthesis of chitin have been identified bioinformatically, with molecular weights of 100 to 130 kDa. The exact biochemical functions of many chitin synthase isoforms remain to be established (33–36), and although some isoforms may have redundant functions, in several species there is evidence that individual chitin synthase enzymes perform distinct and specific functions under normal growth conditions. Although the enzymological product of all chitin synthase enzymes is a homopolymer with only one linkage, individual chitin synthase enzymes can synthesize chitin fibrils of differing architecture, perhaps due to differences in the folding and intrachitin hydrogen bonding of the primary chain (37). Two families of fungal chitin synthases with three classes in the first family (I, II, III) and four classes in the second family (IV, V, VI, VII) have been identified based on amino acid sequence (38). Four classes (III, V, VI, VII) are specific to filamentous fungi (36, 39). The significance of each of these seven classes is not well understood, since mutations in members of a common family do not always result in a similar phenotype. However, two groups of mutants can be identified: the

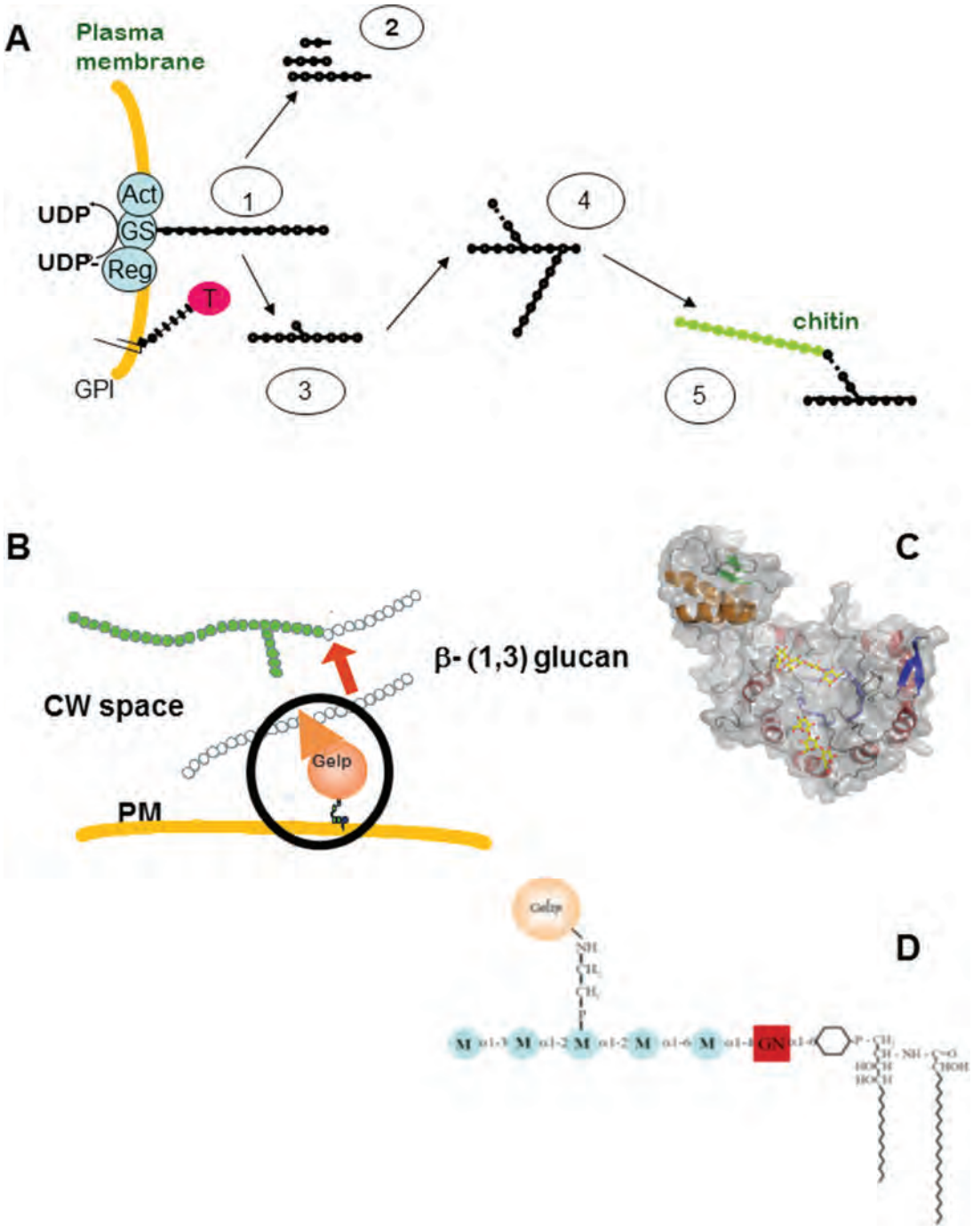


Figure 2 Synthesis and remodeling of β-(1,3) glucan. (**A**) Putative sequential or concomitant events in the synthesis and remodeling of β-(1,3) glucan. 1. Synthesis of linear glucan chains (glucan synthase complex composed of a catalytic [GS], activating [Act], and regulating [Reg] subunits). 2. Hydrolysis of glucans. 3. Branching of β-(1,3) glucan. 4. Elongation of β-(1,3) glucan side chains. 5. Cross-linking with branched [β-(1,3)] glucan. GPI-anchored transglycosidase or hydrolases (T) bound to the membrane can act on the polysaccharides in the cell wall space. Panel A provides example. (**B**) An example of GPI-anchored Gel1 protein involved in the elongation of β-(1,3) glucan inside the cell wall space. (**C**) Crystal structure of the *S. cerevisiae* Gel1 orthologue, Gas2 complex with acceptor and donor oligosaccharides. The enzyme is shown as a ribbon, the glucan binding domain with green strands and orange helices, and the catalytic domain with blue strands and red helices. A gray transparent molecular surface is shown, revealing an elongated groove on the catalytic domain, in which the laminarioligosaccharides (shown as sticks, with yellow carbon atoms) bind. (**D**) Biochemical organization of a GPI-anchored protein in *A. fumigatus*. The three domains of the GPI anchor are (i) a phosphoethanolamine linker covalently bound to the protein, (ii) a mannan-glucosamine-myo-inositol oligosaccharide, and (iii) a ceramide tail attaching the GPI anchor to the cell membrane. (Data from reference 86).

first has reduced chitin content but normal chitin synthase activity *in vitro*, whereas the second is affected in enzyme activity but has regular cell wall chitin content. In addition, other genes, named *CHS*, are not associated with chitin synthase activity but are involved in the regulation of chitin synthase activity or localization. Some chitin synthases appear to be zymogens that are activated by proteolysis, and there is also evidence that some chitin synthase enzymes are regulated by phosphorylation (40, 41). Some fungi have more than 20 *CHS* genes, and some have only 1 (42). *A. fumigatus* and *C. neoformans* are both predicted to have eight *CHS* genes, and *Wangiella dermatitidis* has five; all of these *CHS* genes are nonessential, although the *chs3* mutant of *C. neoformans* cannot grow at 37°C. In contrast, *C. albicans* has a *CHS* family of four genes, and the class II *CHS1* is essential for cell viability (43). In *Aspergillus* species, some double chitin synthase mutants are lethal, but most single mutants are viable (39).

The class V and class VII chitin synthase enzymes of filamentous fungi have unconventional myosin-motor-like domains (MMD) (44). These enzymes are often essential for growth, morphogenesis, and virulence, as well as stress tolerance. It is likely that the MMD functions in actin-mediated cytoplasmic transport, and there is evidence for this domain's ability to bind actin and to influence apical localization (45). However, this domain is not required for cellular motility in *Aspergillus nidulans* and *Ustilago maydis*, and (46) instead the MMD may function in tethering vesicles in the apical dome, increasing the residence time at that location, and thereby favoring vesicle fusion with the PM (47).

β-(1,3) Glucan, the other major cell wall polysaccharide, is synthesized by a PM-bound glucan synthase complex which uses UDP-glucose as a substrate and extrudes linear β-(1,3) glucan chains through the membrane into the cell wall, where it can act as a substrate for various transglycosidase enzymes (see below) (48). The protein complex contains at least two proteins: (i) the putative catalytic subunit encoded by the gene(s) *FKS*/*GSC* and (ii) a regulatory subunit encoded by *RHO1* with an M_r of ~20 kDa. The Fks/Gsc subunits are the target of the echinocandin family of antifungal drugs and the recently described plant metabolite poacic acid (49, 50). Fks subunits have an M_r of >200 kDa with up to 16 transmembrane helices and a central hydrophilic domain of about 580 amino acids, which displays a remarkable degree of identity (>80%) with all known Fks proteins (48). Two external loops of Fks contain so-called hot-spot regions: sites in which common mutations confer reduced sensitivity to echinocandins (51). Rho1-GTPase is regulated by switching between a GDP-bound inactive state to a GTP-bound active state with accompanying conformational changes (1). There are fewer glucan synthase genes than chitin synthase genes in pathogenic fungi. In *A. fumigatus* and *C. neoformans*, *FKS1* is unique and essential (52). In *C. albicans*, three *FKS* orthologues have been identified, but only one of them, *orf19.2929*, is associated with echinocandin resistance (53).

Recently, it was shown in *U. maydis* that the class VII chitin synthase Mcs1 and the class V chitin synthase 6 (Chs6) can be cotransported on the same secretory vesicle along with the β-(1,3) glucan synthase Gsc1 (Fig. 3) (45). Moreover, the cocomplex of glucan and chitin synthases seems to be retained at a localized spot of exocytosis and wall synthesis by the tethering effect of the synthases to their nascent polysaccharides that reside within the fabric of the external cell wall (45).

Biosynthesis of Mannan and Other Decorating Polysaccharides

The yeasts *S. cerevisiae* and *C. albicans* have an outer layer of proteins that are highly glycosylated with α and β-linked oligomannosyl residues by mannosyltransferases that use GDP-mannose as a substrate. *N*-glycans are the major form of mannoprotein modification and consist of a core structure, which is similar in all eukaryotes and is further elaborated in the Golgi to form an outer chain comprising a linear α-(1,6) mannan backbone that is highly branched with α-(1,2)- and α-(1,3)-containing side chains (54, 55). In some species these may be further modified with mannosyl phosphate that may contain β-(1,2) mannan. *O*-Linked mannans of fungi tend to be short, linear chains composed of α-linked mannose sugars. In *A. fumigatus* and other molds, long mannan chains are also bound to core polysaccharides (9), and mannosyl groups also form part of GPI anchors. Although the mannan structural organization of fungi can differ substantially, a comparative genomic study has indicated that orthologues of most yeast mannosyltransferase genes can be found in the genome of *A. fumigatus* and other filamentous molds, although other branched chain mannans do not seem to be represented in the genome of *Pneumocystis* and possibly other fungi (56). In *A. fumigatus*, deletion of 11 genes coding for putative mannosyltransferases had little effect on the growth or physiology of *A. fumigatus* (57).

The synthesis of other decorating polymers remains less well understood. For example, in the case of α-(1,3) glucan synthesis, only the genes encoding putative α-(1,3) glucan synthases have been identified (13, 58). They are the largest known genes (~8 kb) involved in

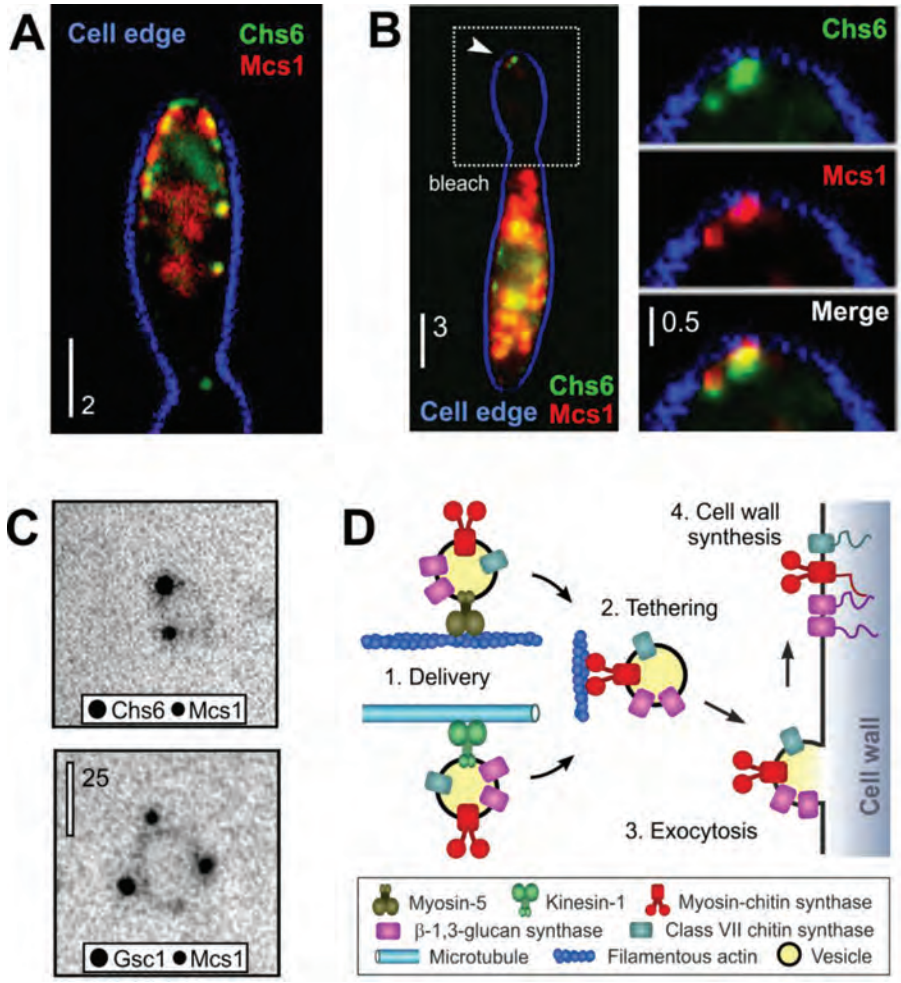


Figure 3 Glucan synthase (Gsc1), chitin synthase (Chs6), and myosin chitin synthase (Mcs1) of *U. maydis* are codelivered on the same secretory vesicles and colocalize at bud and hypha tips. (**A**) mCherry3-Mcs1 (red) and Chs6-GFP3 (green and yellow) colocalized Mcs1 and Chs6 at the bud tip. Scale bar, 2 μm. In (**B**) the bud is photobleached with a laser, and the codelivery of mCherry3-Mcs1 (red) and Chs6-GFP3 (green) into the photobleached bud is revealed after 5 minutes. Scale bars, 3 μm (left) and 0.5 μm (right). (**C**) Electron microscopy of secretory vesicles that have been colloidal-gold-labeled with antibodies showing Chs6 and Mcs1 colocalization in a single vesicle. Scale bars: 100 nm. (**D**) A model for the delivery and secretion of vesicles containing both Chs6 and Msc1 via actin- and microtubule-based cytoplasmic transport systems to the apical cell membrane. After fusion with the apical membrane, the nascent polysaccharide chains of chitin and β-(1,3) glucan are inserted into the cell wall—a process that anchors the synthases *in situ*, ensuring coordinated synthesis and tethering at the biosynthetically active apical region of the cell. (From Schuster et al. [45], with kind permission and modification by Gero Steinberg.)

cell wall polysaccharide synthesis and are characterized by two putative hydrolase and synthase domains separated by a single transmembrane domain. Deletion of a single α-(1,3) glucan synthase gene (NcAGS-1) or multiple genes (AGS1, AGS2, and AGS3) generates defects in the conidial cell wall in *Neurospora crassa* and *A. fumigatus*, respectively (59, 60), without impacting the growth of the vegetative mycelium. Deletion of the three *AGS* genes in *A. fumigatus* also resulted in attenuation in virulence in a mouse aspergillosis model. Similarly, genes involved in β-(1,6) glucan synthesis have been identified based on the resistance of mutants to the K1 killer toxin, which kills yeast by binding to β-(1,6) glucan (61). Many of these *KRE* genes such as

KRE2, *KRE5*, *KRE6*, and *KRE9* impact glucan synthesis without being directly associated with an enzymatic activity (9, 62). Permeabilized *S. cerevisiae* cells are capable of synthesizing β-(1,6) glucan when supplied with UDP-glucose, and the amount of product was reduced in the absence of Kre5 or Kre9 (61, 63–65). Deletion of *KRE5*, *KRE6*, or *SKN1* in *C. neoformans* provided evidence that these genes are also involved in β-(1,6) glucan synthesis in this pathogen and that the mutations also affected capsule formation, chitosan levels, and retention of cell wall mannoproteins (66).

In *A. fumigatus*, the UDP-glucose 4-epimerases Uge3 and Uge5 are required for synthesis of UDP-galactopyranose. Galactofuran side chains of GM are synthesized by the sequential action of the UDP galactose mutase Ugm1 and the galactofuranosyltransferase Gfsa. More recently, a cluster of genes has been implicated in the biosynthesis of the galactosaminogalactan GAG in *A. fumigatus* (67). This cluster contains the *ADG3* gene encoding a protein with a deacetylase domain, which deacetylates GAG, giving it polycationic properties, which are required for it to adhere to the hyphal surface and for biofilm formation. GAG has been shown to be an important virulence factor of *A. fumigatus* responsible for conidial adherence to epithelial cells as well as man-made surfaces (68, 69), with anti-inflammatory effects in mice (70).

Melanin

Melanins are negatively charged hydrophobic pigments of high molecular weight that are composed of polymerized phenolic or/and indolic compounds (71, 72). Little is known about the detailed structure of melanin mainly because of the lack of suitable technologies to analyze amorphous, insoluble materials that are resistant to harsh chemical treatments. Indeed, most of the structural information about melanin comes from molecular studies deciphering the melanin metabolic pathways. Two main types of melanin are found in the fungal cell wall: the DHN-melanin of *Aspergillus* species and black fungal pathogens such as *W. dermatitidis* or *Sporothrix schenckii* and the 3,4-dihydroxyphenylalamine (DOPA)-melanin found in *C. neoformans*. Enriching growth medium with L-DOPA has even been shown to induce melanin production in *C. albicans* (73). A third form of water-soluble pyomelanin whose functional significance is less clear also exists in some fungi and bacteria.

Melanin in *C. neoformans* is synthesized by a laccase located in the outer layer of the cell wall in the presence of DOPA. A model structure has been established in which the concentric melanin layers in the wall come from irregularly shaped melanin granules. The spaces between granules would determine the size of the pores controlling the passage of different secreted molecules (74). In addition, solid-state nuclear magnetic resonance data have identified a putative covalent linkage between melanin and mannose-containing polysaccharide motifs, suggesting that melanin may be anchored to the cell wall via linkages with galactoxylomannan or mannosylated proteins. Other work also suggests associations of melanin with chitin or chitosan (73, 75).

DHN-melanin (named from one pathway intermediate 1,8-dihydroxynaphthalene) is formed from malonyl-CoA by the action of several enzymes including a polyketide synthase and several reductases and dehydratases. Deletion of the genes encoding the polyketide synthase (alb1 = pksP) of *A. fumigatus*, the initial step in the pathway, results in the production of conidia with a variety of different colors (76). In contrast to DOPA-melanin, no microstructure of cell wall-associated DHN-melanin has been obtained. Nor is it understood how the putatively intracellularly synthesized melanin crosses the cell wall barrier to become immobilized on the conidial surface. In addition, DOPA-melanins can be formed by *Aspergillus* and other black fungi (77, 78). Based on studies of plant pathogenic fungi, it can be predicted that the structural role of melanin in human fungal pathogens is to increase cell wall rigidity, enabling hyphae of black fungi such as *W. dermatitidis* to penetrate host tissues and pigmented conidia of *Aspergillus* or yeast cells of *Cryptococcus* to remain turgid when desiccated.

Cell Wall Proteins

There are multiple classes of proteins in the cell wall of fungi, whose functions are diverse and sometimes species-specific. They can serve in modifying the properties of the wall, in adherence to surfaces, and in protecting the fungus from harmful environmental elements or disguising it from phagocytes.

GPI proteins

The complement of cell wall-associated proteins and, in particular, those predicted to be GPI-anchored appear to be rapidly evolving, with a number of species-specific proteins evident (4, 79). Genome-wide analyses have been performed to predict all proteins that can be modified by the addition of a GPI anchor (80, 81). While *S. cerevisiae* is predicted to have around 66 GPI proteins, many fungal pathogens have many more, with some *Candida* species having well over 100 predicted GPI proteins (79, 80). Within this cohort in *Candida*

are a number of families, such as the Als, Iff, Epa, Sap/yapsins, and Sod proteins. These families show marked variation both in the number of members and in the case of the Als and Iff families in the number of intragenic tandem repeats within family members between and within different species (80–82). Furthermore, many predicted GPI proteins are species-specific, with no known orthologues (80). Whether the diversity among the surface proteins has consequences in terms of relative pathogenicity of the different species remains to be firmly elucidated, because many species-specific GPI proteins have unknown functions.

In *C. albicans*, many of these *CWP* genes are highly regulated at the transcriptional level. Some are regulated during yeast-to-hypha morphogenesis, during the response of *C. albicans* to various environmental changes and stresses and, presumably, *in vivo* during the establishment of *C. albicans* infections (83). Non-gel proteomics using tandem liquid chromatography-mass spectrometry/mass spectrometry is now providing a global view of the cell wall proteome. It is possible not only to detect which proteins are cell-wall-localized (82, 84) but also to quantify them (85). This approach has identified 15 to 21 cell wall proteins on the surface of *C. albicans* when grown under rich laboratory culture conditions. Altering the environmental conditions such as carbon source, iron limitation, or hypoxia has a direct effect on the cell wall proteome composition and the abundance of certain wall proteins (86–88). We still have little knowledge of cell wall protein expression, both quantitative and qualitative, during infection. Although many cell wall proteins are known to be immunogenic and hence likely to be expressed *in vivo* and to play roles in adaptive immunity, their significance in growth and pathogenesis is not known (89).

In most natural situations, the outer cell wall is impregnated with, or loosely attached to, a greasy outer layer of cell wall-associated proteins, sometimes called "moonlighting proteins." Much has been made of the apparent conundrum that these proteins are predominantly of cytoplasmic origin such as enolase, collagen, translation elongation factors, and certain heat shock proteins, which do not have a signal sequence for export across the cell membrane (86, 90, 91). Such proteins are readily removed by extraction protocols using SDS and reducing agents such as β-mercaptoethanol and dithiothreitol, which had been thought mild enough to preserve the integrity of the cell (91). However, it has been demonstrated that such treatment may partially solubilize the membrane, leading to the leakage of cytosolic contents (91). Biotinylation of cell wall proteins can also potentially permeabilize the cell membrane at moderate temperatures (91). Therefore, the likelihood is that most of these cell wall-associated proteins leak out of cells and become incorporated at the cell surface when cells are treated with reagents that semipermeabilize the cell membrane.

It is clear that the cell walls of fungi such as *C. albicans* have a significant capacity to absorb soluble proteins from the environment and that fungal surfaces are normally contaminated with cytoplasmic proteins that are picked up from the environment. Alternatively, it is possible that exosomes (secreted vesicles that transit intact through the cell wall) deliver cytoplasmic proteins to the surface, bypassing the normal secretory pathway (92). Once deposited on the cellsurface, some of these proteins can impart important properties such as the binding of plasminogen and other host proteins (93), or opsonization, and could therefore have a direct effect on the function of the cell wall. In *C. albicans*, the hydroperoxide peroxidase-like protein Tsa1p is bound specifically to hyphal cells despite being expressed at approximately equal concentrations in yeast and hyphal cells (90, 94). This suggests that different cell surfaces are differentially receptive to protein binding and that the composition of cell wall-associated proteins could vary substantially between and within species and different cell types.

Transglycosidases

While most of the cell wall biosynthetic processes occur in the Golgi and at the cell membrane, part of the biosynthesis of the fungal cell wall takes place within the wall itself. Neosynthesized polysaccharides are linear or amorphous and become cross-linked to other polysaccharides by transglycosidases that are putatively anchored to the PM or located in the cell wall space to form the rigid three-dimensional network typical of the cell wall (Fig. 2B,C). The first transglycosidase identified as contributing to cell wall organization is a GPI-anchored enzyme encoded by the genes *GEL* and *PHR* in *Aspergillus* and *Candida* and *GAS* in *Saccharomyces* that splits internally a β-(1,3) glucan molecule and transfers the newly generated reducing end to the nonreducing end of another β-(1,3) glucan molecule (95, 96) (Fig. 2B). The generation of a new β-(1,3) linkage between the acceptor and donor molecules results in the elongation of β-(1,3) glucan chains. Transglycosidases that play such an essential role in branching and cross-linking polysaccharides should be common to all fungal species. Comparative genomic and proteomic analyses of ascomycete fungal species have identified

six families of conserved, GPI proteins: Sps2, Gas/Gel, Dfg, Plb, Crh, and Yps (79, 96). The Sps2 and Dfg5 families are involved in cell wall construction, and the Crh family is involved in cross-linking β-(1,6) glucan and chitin (97–99).

Cell wall hydrolases and deacetylase plasticizing the rigid cell wall

There is clear evidence that endo β-(1,3) glucanases and chitinases participate in cytokinesis since mutations in these genes and inhibitors of these enzymes affect the separation of mother and daughter cells (100, 101). In filamentous fungi, which do not undergo cytokinesis, some models of cell wall synthesis invoke a delicate balance between cell wall synthesis and hydrolysis at the hyphal apex (102). However, there is no unequivocal evidence that cell wall hydrolases are required for tip growth, and mutants in *C. albicans* and *A. fumigatus* with single and multiple mutations in chitinase and endo β-(1,3) glucanase genes do not appear to differ in growth rate or hyphal morphogenesis (103, 104).

Similarly, in zygomycetes, ascomycetes, and basidiomycetes substantial deacetylation of chitin to chitosan occurs, creating a more flexible molecule that becomes resistant to chitinases. Chitin deacetylase genes have been identified, but in general, their role in fungal morphogenesis is not clear yet. In *C. neoformans*, disruption of all three chitin deacetylase genes (105) attenuates virulence and results in a defect in cell wall integrity. In plant pathogens, chitin deacetylation can prevent plant receptors from recognizing chitin of plant pathogens (see below).

Yapsins

The yapsins play important roles in cell wall remodeling and in maintaining a robust cell wall. They comprise a subset of the aspartyl proteinase family, and in contrast to other members that are secreted, they are tethered to the membrane and the wall via the addition of a GPI anchor. The cell wall-localized proteolytic activity of ScYps1 was shown to be pH-regulated, and it was shown that Yps1 acted as a "sheddase," releasing a number of GPI proteins from the wall—notably itself and Gas1 (106). In *C. albicans*, deletion of both the yapsin genes *SAP9* and *SAP10* resulted in reduced adherence to epithelial cells and in a reduction in epithelial damage in a reconstituted human epithelial model of oral infection (107). Similarly, *Candida glabrata* has a family of eight yapsins that have been implicated in virulence as well as in maintenance of cell wall integrity (108).

Adhesins

One vital property of the fungal cell wall that promotes virulence is adhesion to host cells and tissues. Several cell wall proteins have adhesin-like properties (86, 109). The best characterized are the *C. albicans* Als family of eight proteins (110) and the *C. glabrata* Epa family (111), both comprising GPI proteins. A more extensive family of GPI-anchored adhesin-like proteins has been identified in *C. glabrata* (112). Both the Als and Epa families have a characteristic domain organization with N-terminal adhesin domains that impart specificity of host protein/glycan binding to different family members (113–116). The *C. albicans* hyphal-specific cell wall protein Hwp1 aids adherence to oral epithelial cells by acting as a substrate for host transglutaminases (117). Both Hwp1 and the Als family play significant roles in biofilm formation, and complementary binding between these adhesin types enhances the attachment of *C. albicans* cells to each other during biofilm generation (118). Interestingly, in pathogenic molds, proteins do not seem to act as adhesins, and this role seems to be a function of cell wall α-(1,3) glucans and GAG (67, 69).

In *C. albicans*, a number of other cell wall proteins have also been shown to contribute to biofilm formation, including Eap1 (119), Sun41 (120–122), and members of a family that contains a CFEM-like domain (Rbt5, Pga10/Rbt51 and Csa1/Wap1) (123).

Hydrophobins

Hydrophobins form a class of amphipathic proteins that can self-assemble to form rodlets, generating a hydrophobic interface between filamentous fungi and their environments (124). The rodlets resemble amyloid fibrils and form a monolayer around aerial structures such as hyphae and fruiting bodies, coating hydrophilic surfaces to make them hydrophobic (18, 19). Hydrophobins play roles in morphogenesis, may be developmentally regulated (125), and are also involved in adhesion of fungal cells to surfaces and hence have been associated with virulence of fungal plant and insect pathogens (126–128). In human pathogens only RodA and RodB, the hydrophobins present on the *A. fumigatus* conidial surface, have been characterized in detail (19). RodA contributes to pathogenesis by protecting conidia from alveolar macrophage killing (20). This rodlet layer has also been shown to be an immune-shield masking the detection of conidia or a range of molds by macrophages and dendritic cells (21). Detection only occurs once the spores swell and germinate—a process that leads to cracking of the hydrophobin layer and hence revealing of the underlying immunologically active glucans.

REGULATION AND SIGNALING

The Cell Wall Salvage Response

The cell wall can be built in different ways depending on environmental conditions and exposure of agents that induce cell wall damage. The integrity of the β-(1,3) glucan-chitin cell wall scaffold must be monitored and regulated constantly to enable growing walls to remain plastic enough to allow turgor-driven cell expansion yet robust enough to prevent bursting of the cell. It is not fully understood how this delicate balance between the rigidity and the compliance of the cell wall is maintained, but it is known that the nascent cell wall at the hyphal apex is thinner, has less hydrogen bonding between the antiparallel α-chitin chains, and has fewer cross-links between chitin and β-(1,3) glucan than the mature wall of the parallel sides of the hypha (102).

Regulation of cell wall biosynthesis occurs at many levels ranging from availability of substrate for biosynthetic enzymes to protein phosphorylation. A number of signaling pathways have been implicated in the regulation of cell wall biosynthesis and in the maintenance of a robust wall (Fig. 4). The pathways often impinge on different elements of the same promoter, allowing fine-tuning of gene expression.

The key pathway that controls cellular integrity via maintenance of the cell wall is the protein kinase C pathway (129) (Fig. 4). Best characterized in *S. cerevisiae*, this pathway is conserved in most fungal species, including human pathogens (130). Highly glycosylated integral membrane sensors, Mid2, Wsc family, and Mtl1, sense perturbations in the cell wall, and ScWsc1 has been shown to act as a nano-spring detecting wall stretching to trigger a response by activating Rho1. This GTPase relays signals to protein kinase C (Pkc1) and also regulates actin polymerization, polarized secretion, and glucan synthesis (129). Pkc1 lies at the top of a mitogen-activated protein kinase (MAPK) cascade and phosphorylates the Bck1 MAPKKK in addition to a number of other substrates, including chitin synthase (41). The signal passes down the MAPK cascade via a phosphorylation relay and ultimately activates transcription factors that regulate target gene expression.

Other pathways that play significant roles in the regulation of cell wall biosynthesis are the Ca^{2+}/calcineurin pathway; a second MAPK cascade, the HOG pathway; and the pH-sensing RIM101 pathway (131). Activation of cell wall compensatory or salvage mechanisms often results in elevated chitin levels in the cell wall and an increase in the number of GPI proteins that are covalently attached to chitin rather than β-(1,3) glucan, reflecting significant alterations to cell wall architecture (132). Transcript profiling experiments in *S. cerevisiae* in various cell wall mutant backgrounds and in cells treated with cell wall perturbing agents have identified a core set of regulatory genes including Rlm1, Crz1, SBF (Swi4/Swi6), Msn2/Msn4, Ste12, and Tec1 that are activated upon cell wall assault (133). Some orthologous genes have also been identified as upregulated in *C. albicans* in response to caspofungin (134, 135), Ca^{2+} (136), and cell wall mutations.

Activation of cell wall salvage pathways, specifically the protein kinase C pathway, is one of the responses to sublethal doses of the echinocandin antifungal drugs (137, 138). Synergism has also been shown between immunosuppressive drugs that block the Ca^{2+}/calcineurin pathway (FK506, cyclosporine A) and the echinocandins in *C. albicans*, *C. neoformans* (139), and *A. fumigatus* (140). Mutants blocked in the protein kinase C pathway, Ca^{2+}/calcineurin, and certain steps of the HOG pathway (but not Hog1) are hypersensitive to echinocandins (138, 140, 141). Echinocandin hypersensitivity has been used as a screen to identify signaling components that are involved in the response of *C. albicans* to this class of antifungal (134, 135). These screens identified a novel *C. albicans*-specific transcription factor, Cas5 (134), and Sko1, the transcription factor thought to be downstream of the Hog1 MAP kinase. Interestingly, Hog1p itself was not required for the echinocandin response via Sko1, but the Sko1-dependent activation of genes induced by caspofungin was dependent on another protein kinase, Psk1 (135).

Regulation of Septation

Redundancy exists in the strategies employed to assemble the fungal cell wall under both normal and cell wall stress conditions (Fig. 5A). This is also evident in the observation in *S. cerevisiae* that a septum can be fabricated in mutants that lack enzymes that are required to make the normal septum. A greatly thickened salvage septum can be made by the Chs3 enzyme in the absence of the ScChs2 chitin synthase that normally synthesizes the chitinous primary septum (33). In *C. albicans*, a septum can still be formed that permits cell division in a mutant that lacks both CaChs3 and CaChs1 (the orthologue of ScChs2), the normal chitin synthase machinery required for septation (138, 142) (Fig. 5B).

Three distinct types of salvage septa were identified in *C. albicans* that could be synthesized in the absence of Chs1 by different combinations of Chs2, Chs3, and Chs8 (142). An implication of this work is that all four chitin synthases in *C. albicans* can be employed for septum formation—a prediction supported by observations

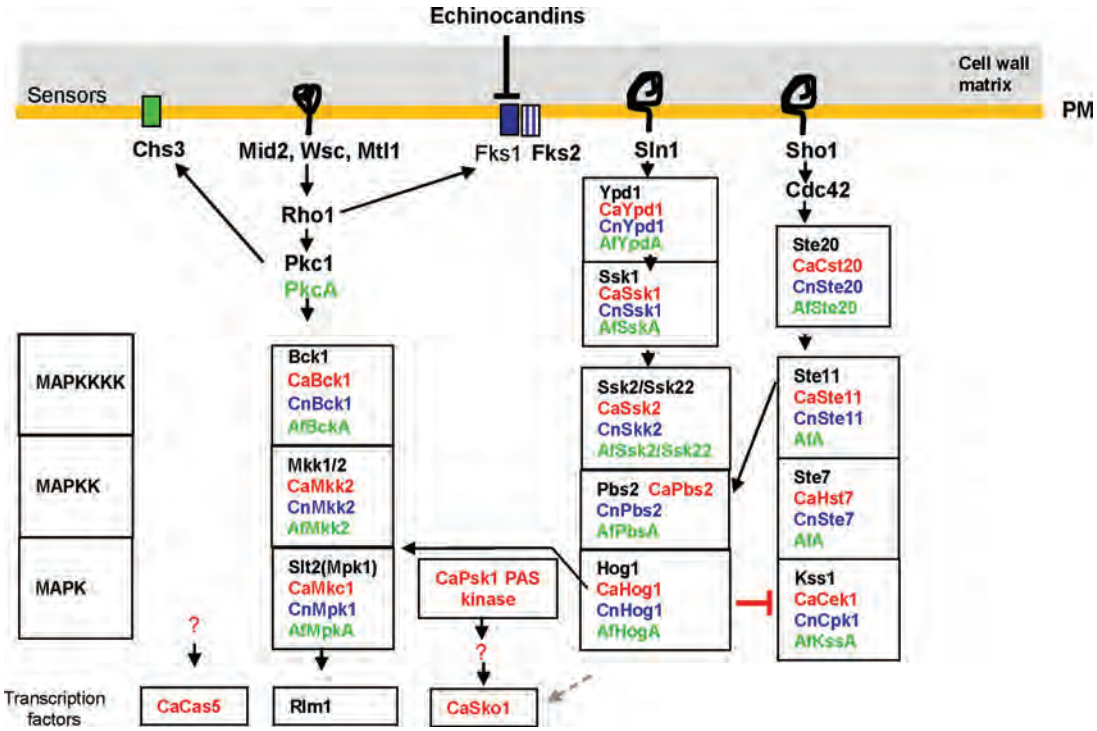


Figure 4 Signaling pathways that regulate cell wall remodeling and cell integrity. Integral, glycosylated, membrane sensors (Wsc family, Mid2, Mtl1, Sho1, and Sln1) detect specific perturbations in the wall and transduce the signal to the downstream pathway elements that feed into MAP kinase cascades. Transcription factors at the bottom of the pathway activate gene expression to promote remodeling of the cell wall architecture to maintain cell integrity. In *S. cerevisiae*, Pkc1 is involved in targeting Chs3 to the plasma membrane in response to heat shock, and Rho1 activates the Fks1 subunit of β-(1,3) glucan synthase. Black text denotes *S. cerevisiae* proteins; red, *C. albicans*; blue, *C. neoformans*; and green, *A. fumigatus*. The fungal pathogen orthologues may not have been fully characterized, and their position in the pathways reflects the *S. cerevisiae* paradigm. However, significant rewiring of signaling pathways is evident in *C. albicans*; for example, the role of the CaSko1 transcription factor in response to caspofungin is independent of the Hog1 MAP kinase (135) but involves the Psk1 PAK kinase. Furthermore, in *C. albicans*, there is no evidence of Ste11 activating Hog1 like there is in *S. cerevisiae* (213). In *C. albicans*, the Cas5 transcription factor also contributes to the transcriptional response to caspofungin, and there are no Cas5-orthologues in *S. cerevisiae* (134). The CaCek1 MAP kinase is also implicated in cell wall remodeling and is constitutively activated in a *hog1* null mutant background (213). Fungal pathogen orthologues of the elements upstream of the MAP kinase cascades are not shown but exist, although the membrane sensors appear to have significantly diverged. Exogenous calcium enters cells primarily through the Cch1/Mid1 channel complexes. A third Ca^{2+} channel, Fig1, plays a role in Ca^{2+} transport during mating, but no orthologues of Fig1 have been identified in *C. neoformans* or *A. fumigatus*. Ca^{2+} binds to and activates calmodulin (Cmd1), which in turn activates the phosphatase calcineurin, composed of a catalytic (Cna1) and a regulatory (Cnb1) subunit. *S. cerevisiae* has two Cna1 isoforms (Cna1/Cmp1 and Cna2/Cmp2). Calcineurin activates the transcription factor Crz1 by dephosphorylation to induce expression of genes that contain calcium-dependent response elements within their promoter sequences. No Crz1 orthologue has been identified in *C. neoformans*. Some data also suggest that calcineurin has regulatory functions that are independent of Crz1 (136). Several of the *A. fumigatus* proteins that may be related to this pathway remain unannotated, so putative orthologs have been ascribed but have not been experimentally validated. The pathway can be blocked via FK506 binding to Fpr1 or cylosporin A binding to cyclophilin Cpr1, and both interactions result in calcineurin inhibition. (Adapted from references 129, 130, 214–216).

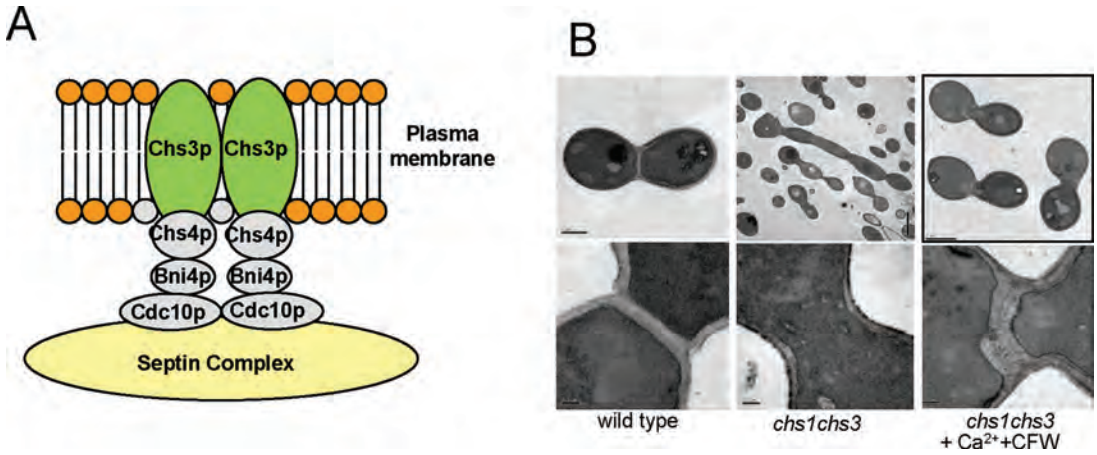


Figure 5 Chitin synthesis and septum formation in yeasts. (**A**) Septation involves a protein scaffold that tethers the Chs3p chitin synthase that assembles the chitin ring to Cdc10p of the septin ring complex via Chs4p and Bni4p. (**B**) The structure of the wild-type septum of *C. albicans* (transmission electron microscopy image on right) is shown alongside septumless yeast cells in a *chs1 chs3* conditional mutant (middle transmission electron microscopy image) and salvage septa (transmission electron microscopy image on left) made in the same mutant strain after stimulation of the cell wall salvage pathways by growth in the presence of calcium ions and calcofluor white. (Reused from reference 138 under CC BY 4.0).

that CaChs1, CaChs2, CaChs3, and CaChs8 are all located at the site of cytokinesis under normal conditions (37, 143). Similarly, all eight chitin synthases localize to the septa of *Ustilago* (143). In *S. cerevisiae*, the septum is assembled on a complex scaffold of proteins that are linked in turn to the septin rings (144) (Fig. 5A). This scaffold involves Bni4p, which tethers the Chs3 chitin synthase enzyme to the mother-bud neck by forming a bridge between a regulatory protein Chs4 and the septin Cdc10. In *C. albicans*, *BNI4* was shown not to be essential for chitin ring formation, but null mutants were affected in bud formation, suggesting that some, but not all, features of this scaffold are conserved between these two species (145). It remains to be established how the various salvage septa of fungi are constructed and how and whether they are assembled on a normal septin ring structure. Septins also play key roles in filamentous fungi. For example, they have been shown to play multiple roles in septation, conidiation, the function of clamp connections, and nuclear dynamics (146, 147).

Septation is a complex process tightly coupled to the cell cycle. The transcription factor Ace2 regulates the expression of many genes that orchestrate the process of cytokinesis. Among these are chitinases and glucanases that in some fungi have been shown to aid the septation of daughter cells by digesting the interstitium of the wall between the completed septal structures (100, 148). Fks1 localization and activation require the Rho1 GTPase as a regulatory subunit (1). Chitin synthase phosphorylation may also be involved in regulating its localization throughout the cell cycle (40, 41), but the details of how chitin synthase and other cell wall biosynthetic genes are targeted in the cell cycle remain to be established.

Regulation of Polarized Growth

Polarized cell wall growth requires the concerted activity of the cytoskeleton and cortical patches of membrane proteins that regulate secretory vesicle traffic to the sites of cell wall growth (149–152). In filamentous fungi, cytoplasmic transport must move vesicles and organelles to the apex over long distances. In hyphae this transport occurs in two stages. Vesicles are delivered to the apical surface first via cytoplasmic transport, probably mediated by microtubules, to a vesicle supply center near the apex called the Spitzenkörper (apical body). Subsequently, docking and fusion with the PM are mediated by the "Arp2/3 complex," which organizes apical actin (151), and an "exocyst complex" which is responsible for vesicle docking and fusion (152). An additional group of apical proteins called the "polarisome," which contains the essential Cdc42 Rho GTPase, is responsible for recruitment of actin and other components required for polarized cell growth. This dynamic process therefore involves the secretory pathway, cytoskeleton function, and the activities of multimeric protein complexes that establish

and maintain polarity. In filamentous fungi with chitin synthases that have an MMD, the MMD contributes to the docking process by retaining the enzymes in the apical dome (47). A detailed description of this integrated process is beyond the scope of this review but has been published elsewhere (138, 141–143). However, ultimately, the cell shape and growth of fungi relate to how the vectorial secretion of secretory vesicles is regulated and to the overall composition of the cell wall (152–155).

Most pathogenic fungi are dimorphic (sometimes polymorphic), and the prevailing morphotype existing in the environment is normally different from the invading form. Cell wall composition varies with morphotype, but data in this area are rather scarce. Universal relationships between morphology (for example, spherical or tubular) and cell wall structure and composition do not exist. In the yeast of *C. albicans* or the conidium of *A. fumigatus*, the amount of chitin is reduced in comparison to the mycelium. However, in *Blastomyces dermatitidis* or *Paracoccidioides brasiliensis*, the amount of chitin is lower in the mycelial form (156, 157) than in the yeast form. β-(1,3) Glucan is higher in yeast of *P. brasiliensis* but lower in conidia of *A. fumigatus* than in their respective mycelial stages. β-(1,3) Glucans are present in higher amounts in the conidia of *A. fumigatus* and almost absent in the yeast phase of *B. dermatitidis* and *P. brasiliensis* compared to their respective mycelial stages. β-(1,3) and β-(1,6) glucans are also absent in the biotrophic hyphae of the plant pathogen *Colletotrichum* (158). In *Sporothrix schenkii*, no difference in composition of the structural polysaccharides is seen between yeast and mycelial cell walls. For some fungi such as *C. albicans*, all morphotypes are hyaline; for others such as *A. fumigatus*, conidia are pigmented, whereas the mycelium is hyaline, and for black fungi such as *W. dermatitidis*, all morphotypes contain melanin. Links between morphology and cell wall composition are even more difficult to establish because cell wall composition is not only stage- and strain-specific but is also dependent on culture conditions (159).

CELL WALL AS A TARGET

Antifungal Target

The cell wall is composed almost exclusively of molecules that are not represented in the human body yet are important or essential for fungal growth viability or virulence. As such, the wall is a near ideal target for the design of antifungal drugs for clinical use. Nikkomycins and polyoxins are specific chitin synthase inhibitors of chitin synthases, and although they often potently inhibit enzyme activity in *in vitro* assays, they are not efficiently taken up *in vivo* and consequently are often not effective antifungals (160).

The newest class of clinically used antifungals are the echinocandins, which are fungal secondary metabolites that inhibit β-(1,3) glucan synthesis in the cell wall. Echinocandins have a cyclic hexapeptide core with a lipid side chain that is responsible for their antifungal activity and determines species specificity. Three compounds are in clinical use—caspofungin, anidulafungin, and micafungin—and new agents such as CD101 (Biafungin) are under development. These drugs have proven to be safe and effective, but they are insoluble drugs requiring intravenous administration. Clinical resistance has been shown to be due to the acquisition of point mutations in one of two hotspots in the outer face of the Fks1 β-(1,3) glucan synthase target protein, and the efficacy of the drugs can be offset by the induction of chitin synthesis in the cell wall (see above) (161, 162).

Target for Mammalian Immune System

In recent years it has been increasingly evident that there are conserved aspects in the ways both animals and plants detect and respond to fungal invaders. Indeed, defense mechanisms against fungal pathogens have been discovered in all higher organisms that have been investigated.

In humans and other mammals, the innate immune system has evolved to recognize conserved microbial structures called pathogen-associated molecular patterns (PAMPs) via a range of pattern-recognition receptors (PRRs) on their cell surfaces (Fig. 6). Detailed analyses of this recognition system for fungi have been extensively reviewed and are beyond the scope of this article (7, 163). However, recognition of the fungal cell wall ultimately results in the uptake and killing of fungal invaders by phagocytes and the induction of innate and adaptive immunity. Almost all of the main components of the fungal cell wall can be detected by immune cells, and there are more PRRs that detect fungus-specific PAMPs than for any other class of organism. Mannans and mannoproteins are recognized by mannose receptor and Toll-like receptor 4 (TLR4), phospholipomannan by TLR2, and β-mannosides by galectin-3 (163). β-(1,3) Glucan is recognized by the C-type lectin dectin-1 (163–165), and chitin can be detected by the mannose receptor and by Nod2 and TLR9 (Fig. 6). Chitin and chitosan have emerged as important immmunoreactive polysaccharides, with chitin having anti-inflammatory

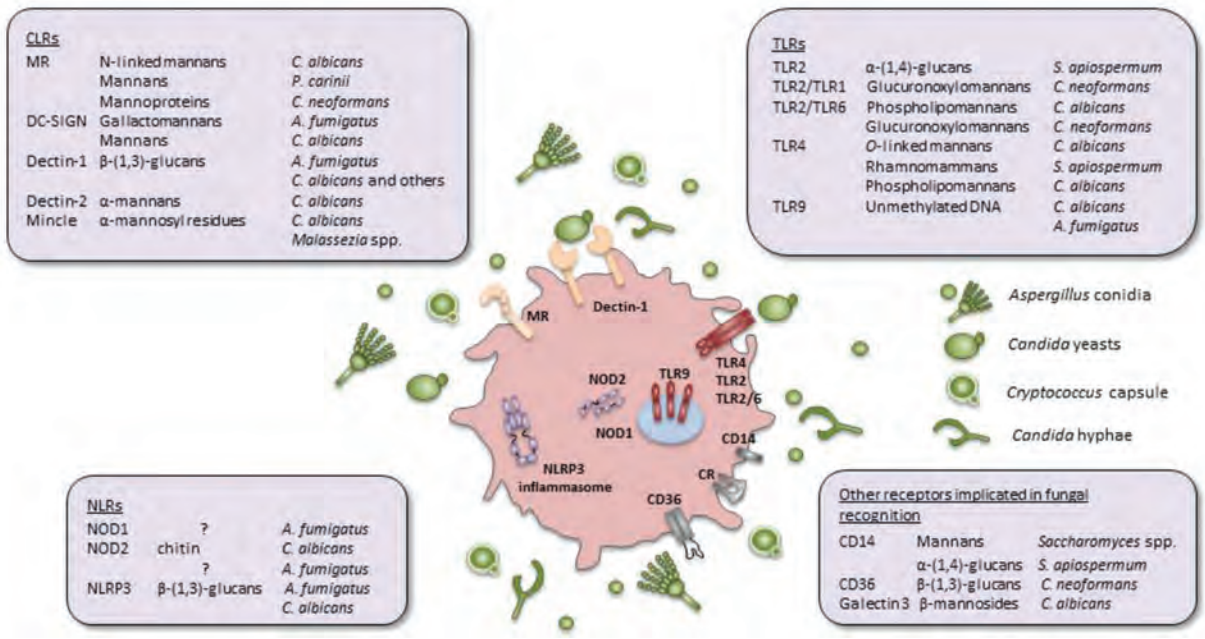


Figure 6 Recognition of human fungal pathogens. PAMP-PRR interactions for fungal cell recognition are shown as described in the text. Interactions with CLRs (C-type lectins), TLRs (Toll-like receptors), NLRs (Nod-like receptors), and a range of other receptors are shown in the purple boxes along with the relevant fungal PAMPs and examples of organisms for which given PRR-PAMP recognition phenomena have been described.

properties, while chitosan is more proinflammatory in nature (166–168). Chitin isolated from *Aspergillus* was shown to have both pro- and anti-inflammatory properties depending on the presence of costimulatory PAMPs, and IgG-opsonized chitin was shown in this study to be recognized by a novel Fcγ receptor-dependent mechanism (169). Chitin recognition is also complicated by the fact that particle size plays an important role in its ability to engage with receptors and induce the secretion of cytokines (170).

Dectin-1 detection of β-(1,3) glucan represents a major recognition mechanism by the immune system. This polysaccharide is often masked by the outer layers of the cell, resulting in shielding of β-(1,3) glucan and escape of immune recognition (7). Various unmasking treatments, such as heat-killing, echinocandin treatment, or genetic deletion of superficial mannans or glucans of several fungal pathogens, have been shown to enhance signaling of leukocytes via dectin-1 (11, 171–173). In the architecture of fungal cell walls, superficial mannoproteins, α-glucans, conidial spore hydrophobins, and melanin can all mask the exposure of β-(1,3) glucan and thereby prevent dectin-1-mediated recognition (Fig. 1) (174). Fungal cell wall PAMPs can be detected singly or in combination by PRP complex leukocytes (175), and the relative importance of individual PAMPs is likely to vary substantially in different immune cell types. It is most likely that recognition of the fungal cell wall involves detection of multiple components of the cell wall that may vary according to fungal species and during different stages and different sites of clinical infections.

Resisting immunity: *Cryptococcus* capsule

More than 40 years ago, it was shown that encapsulated cryptococci are resistant to phagocytosis (176). Capsular polysaccharides are able to scavenge oxygen-related oxidants as well as antimicrobial peptides that are essential for killing phagocyte effectors (177). However, the protective effect of the capsule varies with the phagocyte cell since a recent study shows that *C. neoformans* enters the endolysosomal compartment of dendritic cells and is killed by lysosomal components despite the presence of a capsule (178). However, it is clear that capsular polysaccharides play an essential role in escaping phagocytosis. Moreover, the enlargement of the capsule size associated with *in vivo* growth is essential for fungal survival and replication inside phagocytic cells. Recent observations have shown that *C. neoformans*, and other species of yeasts, can induce

its own expulsion from the phagosome (179, 180). After the expulsive event, both the macrophage and the expelled *C. neoformans* continue to grow normally. This mechanism, which allows the pathogen to escape the phagocyte without triggering host cell death and subsequent inflammation, is entirely dependent on the presence of the capsule since acapsular cells do not promote phagosomal extrusion.

Resisting immunity and stress: melanin

Melanins are an adaptation of fungi to resist environmental stress. Melanized fungal cells resist extreme temperature and UV and ionizing radiation; indeed, melanized fungi in soils have been shown to be able to harvest energy from ionizing radiation for growth (181, 182). Melanin also binds to heavy metals or antifungal drugs, resulting in their detoxification. Internalization by phagocytes of *C. neoformans*, *S. schenkii*, or *F. pedrosoi*, but not *Aspergillus*, can be directly affected by the presence of melanin in the cells. Melanin also protects microbes from host defense reactions since albino strains are more susceptible to killing after phagocytosis. Resistance is due to quenching of nitrogen- or oxygen-derived radicals (67) or microbiocidal peptides (67, 183). After phagocytosis, the only material left is the melanin ghost, confirming the extreme resistance of this compound not only to chemical treatments but also to immunological aggression. These data explain why melanin confers a survival advantage for the melanized morphotypes in the environment and especially to enzymatic degradation by the surrounding hostile microflora.

Target for the Plant Immune System

Plant immunology is now a well-established discipline, and it is clear that plants also recognize fungal PAMPs to trigger immunity (8, 184, 185). Plant PRRs can be located on the plant cell surface or receptor-like proteins that bind fungal and other PAMPs of damage-associated molecules.

Recognition of chitin plays a major role in plant immunity to fungi. Two major chitin PRRs have been characterized. The chitin binding protein LysM-receptor-like protein CEBiP was first identified in rice, while in *Arabidopsis*, chitin-triggered plasmodesmatal closure is induced by LysM-receptor kinase LYK4 (8, 184, 185) (Fig. 7). Plants and fungi have been engaged in an immunological arms race that mediates recognition and disguise of fungal chitin. Plant chitinase secretion can liberate chitin fragments that promote recognition. Reciprocally, some fungal pathogens synthesize α-(1,3) glucan or secrete effector molecules to block chitin recognition or chitinase-mediated attack (186, 187) (Fig. 7). Some fungi produce high-affinity chitin-binding effector scavengers that bind chitin and prevent its interaction with plant chitin receptors or block chitin-induced chitin receptor dimerization that is required for signaling. Some fungi convert chitin to chitosan to escape chitinase degradation and prevent recognition by chitin receptors of the plant immune response (Fig. 7) (188).

An excellent example comes from a study of the hemibiotrophic plant pathogen *Colletotrichum graminicola*, which infects maize. The invading biotropic hyphae of this fungus coordinately downregulates *GLS1*, *KRE5*, and *KRE6*, resulting in the formation of hyphae with little or no exposed β-(1,6) and β-(1,3) glucan (158, 188). They also induce chitin deacetylation in the hyphal walls. As a result, the fungus can colonize the plant tissue without inducing PAMP-triggered immunity.

Diagnostics and Immunotherapeutic Targets

Promising vaccines and immunotherapies are under development that are mainly based on cell wall or cell wall-associated components (189–192). Examples that have been shown to be efficacious in mouse models of systemic mycoses include *C. albicans* vaccines based on the N-terminal regions of Als1 (193) and Als3 (194), synthetic glycopeptides that are based on epitopes of cell wall-associated proteins conjugated to mannose trisaccharides (195), and anti-Mp65 antibodies (196). Vaccination of mice with recombinant versions of the allergen Aspf3 protected mice from invasive aspergillosis (197). An antibody against GXM of the *C. neoformans* capsule conveys protection in animal models and has undergone phase I clinical trials (198). An antilaminarin [anti-β-(1,3) glucan] monoclonal antibody can inhibit growth of *A. fumigatus*, *C. albicans*, and *C. neoformans*, suggesting that a single therapy of this type may be efficacious against a wide range of fungi (199–201).

Cationic antimicrobial peptides also play important roles in host defense against microbial pathogens including fungi (202) and have been shown to be important in the oral cavity, lungs, and GI tract. The action of salivary Histatin 7 is mediated through heat shock proteins Ssa1 and Ssa2 (203), which have been shown to be cell wall and membrane associated. Novel therapies based on antimicrobial peptides have much potential in the clinic and can be used, for example, to coat inanimate medical devices such as indwelling catheters to prevent microbial biofilm formation, one of the risk factors associated with invasive fungal infections (204).

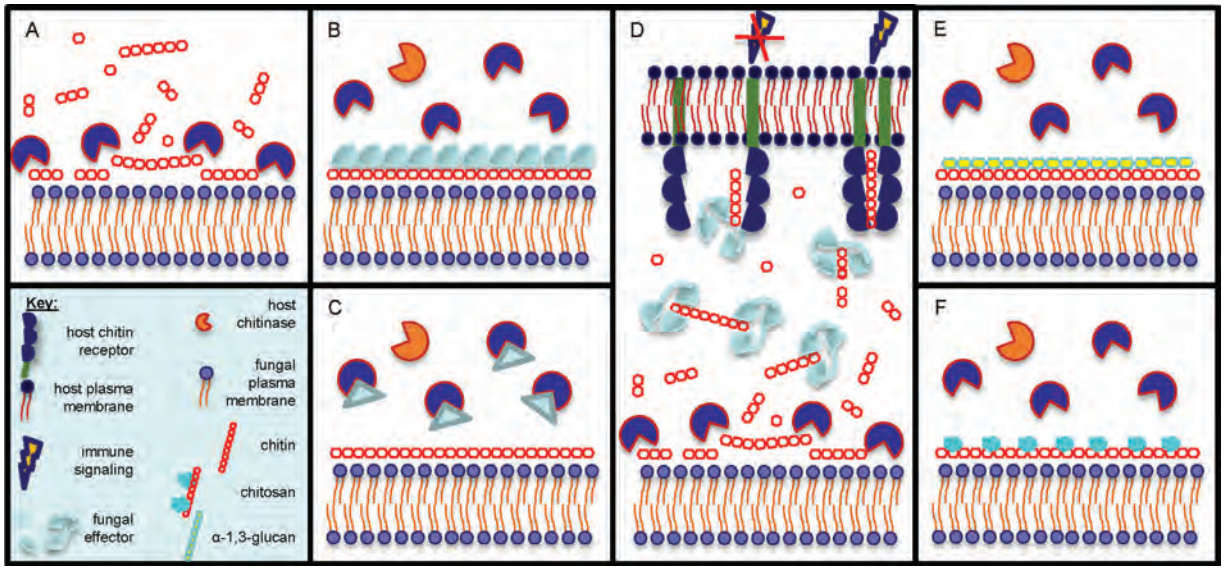


Figure 7 Recognition and avoidance of the recognition of chitin by plant pathogens. The detection of fungal chitin is used to trigger PAMP-mediated immunity in plants. To counter this, plant pathogenic fungi have evolved a range of mechanisms to avoid detection, including the following. (**A**) The liberation of chitin fragments by host chitinase attack can activate host immunity. (**B**) Countering this, some phytopathogens secrete effectors that block access to chitinase or (**C**) inhibit chitinase activity. (**D**) Fungal LysM-type effectors block recognition either by tight binding to prevent engagement with the host PRR or by interfering with host receptor dimerization. (**E**) The synthesis of an outer cell wall layer of α-(1,3) glucan (as in certain human pathogenic species) prevents chitinase action and access to inner cell wall PAMPs. (**F**) Some fungal pathogens convert, to a greater or lesser extent, chitin into chitosan by inducing chitin deacetylases. This modified form of chitin is a poor substrate for chitinase and only weakly induces plant immune recognition. (From Bart Thomma with permission [adapted from reference 186]).

Diagnostic Antigens

Cell wall polysaccharides have formed the basis of the development of serological tests for the diagnosis of systemic fungal infection since their presence in the biological fluids of infected immunocompromised patients is directly correlated to fungal growth. An increase in the circulating antigen results from expanded fungal growth synonymous with a worsening of the disease, whereas successful antifungal therapy or immune reconstitution is associated with a reduction in antigen concentration. Galactomannan, mannan, and glucuronoxylomannan are the specific cell wall polysaccharides of interest for the diagnosis of aspergillosis, candidiasis, and cryptococcosis, respectively.

A monoclonal antibody directed against galactofuranose residues is used for the diagnosis of aspergillosis (205). The sensitivity of the commercially available sandwich enzyme-linked immunosorbent assay test is very high (on the order of nanograms per milliliter). This is due to the presence of epitopes not only on the cell wall polysaccharides but also on glycoproteins and glycolipids of *Aspergillus* species. This MAb recognizes β-(1,5) linked galactofuranose residues but also terminal Gal*f* β-(1,2) linked to mannan of *N*-glycans (206).

Identifying the presence of both α- and β-mannan increases the specificity and sensitivity of candidemia diagnosis. In the case of candidiasis, parallel monitoring of circulating mannan and antimannan antibodies can be performed. A decrease in antibody titer often correlates with an increase in antigen detection (207, 208).

Polyclonal antibodies have been raised against a mixture of complex capsular polysaccharides (GXM) of *C. neoformans* to develop EIA or latex agglutination tests able to recognize all serotypes of this fungal species (209).

A test for the detection of β-(1,3) glucan has also been developed using a modification of the *Limulus* proteolytic cascade identifying a β-(1,3) glucan as an ancestral innate defense reaction undertaken by arthropods. The sensitivity of this test is very low since it can

detect 10 to 50 pg of β-(1,3) glucans/ml (210, 211). All these tests, however, have associated problems with false positives and negatives, insufficient early detection in comparison with clinical signs, and interaction with serum proteins including antibodies that require a proteolytic or EDTA treatment of the serum. In addition, the exact composition of the antigens produced *in vivo* as well as an understanding of the antigen secretion process is often unknown (62, 212).

CONCLUSIONS

The cell wall represents a major organelle of the fungus, determining viability, shape, and interactions with the environment. The assembly of the cell wall involves more than a thousand genes that encode a complex network of metabolic, signaling, and biosynthetic functions. Recent research has shown that redundant pathways exist to activate cell wall salvage mechanisms under conditions of cell wall damage and that the assembly of the wall can be modified and adapted to cope with stress. Although the walls of most fungi have a common skeleton, they also comprise a huge diversity of specialized molecules that are attached to the skeleton that define their specific ecologies. A range of mechanisms exist that ensure that the surface characteristics of the wall are hugely variable, and indeed, it is likely that no two cell walls that are identical have ever been assembled in any fungus. For fungal pathogens, the cell wall remains an ideal yet somewhat unrealized target for chemotherapy since its enzymology is fungus-specific. Similarly, the unique set of fungus-specific polysaccharides and glycoproteins that compose the outer and inner cell wall layers represent fungus-specific pattern recognition molecules and antigens that stimulate immune function of animals and plants. However, the inherent variability of the cell wall means that it is a moving target for both antifungal chemotherapy and for immune surveillance, and a raft of immune recognition avoidance mechanisms has evolved for both plant and animal pathogens that drives a "detect-or-escape" arms race between the host and pathogen.

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Fungal Ecology: Principles and Mechanisms of Colonization and Competition by Saprotrophic Fungi

13

Lynne Boddy and Jennifer Hiscox

INTRODUCTION

Decomposer fungi, by their very nature, continually deplete the organic resources in which they grow and feed. They therefore rely on continual successful spread to new resources. In terrestrial ecosystems resources are distributed heterogeneously in space and time (1, 2). They are often discrete, ranging in size from small fragments, e.g., bud scales, to large tree trunks, though discrete leaves *en masse* can form a continuous layer on the forest floor. The processes of arrival and spread are thus crucial to the success of saprotrophic fungi. Following arrival at a resource, their competitive ability determines whether they are successful in colonization and how long they retain that territory. Colonization and competition are the main focus of this paper and are discussed separately below, largely drawing on wood decay fungi for illustrative examples.

In view of the large number of decomposer fungi, and the variety of organic material that is available for them potentially to feed on, it is not surprising that they have evolved a range of different life history strategies to cope with the environment they inhabit, the three major drivers being stress (S-selected), disturbance (R-selected or ruderal), incidence of competitors (C-selected), or a combination of these (3–5). Characteristics defining these life history strategies are listed in Table 1. These strategies are relative depending on the communities being considered, and they vary in different stages of the life cycle and between regions of the same mycelium exhibiting different physiological states. The wood-decaying basidiomycete *Phlebia radiata*, for example, has a rapidly extending aseptate mycelial margin, which can utilize only simple carbon sources and does not recognize antagonists (R-selected characteristics), whereas the more mature septate mycelium is able to use the lignocellulose complex and is antagonistic to other mycelia (C-selected characteristics) (6). Thus, taxa should not usually be classified *per se* as having a specific life history strategy, but their behavior in a particular context can be defined by these terms. Further, fungi often have combinations of characteristics from different strategies (R-C, R-S, C-S, or R-C-S; Fig. 1).

COLONIZATION

Arrival

Most fungi can only spread between resources by producing asexual spores, sexual spores, or sclerotia. These fungi are termed resource-unit-restricted, as opposed to non-resource-unit-restricted fungi that grow out of resources and spread as mycelium. Spores can enable rapid spread, sometimes over many kilometers (7–11), though most basidiospores, for example, land within a few meters of the basidiocarp that produced them (9, 11). Spores, however, usually contain only small food reserves, and the chance of landing on a suitable new resource with an appropriate environment for germination and growth is small if spread by wind or rain, though greater if transported by animal vectors (5, 12). Sclerotia often contain larger food reserves and allow survival in time, though spread is more limited than for most other spore types (13). Thick-walled chlamydospores also enable survival in time, e.g., the wood-decaying basidiomycetes *Botryobasidium* spp.,

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Table 1 Characteristics defining the life history strategies of ruderal, combative, and stress-tolerant species

Characteristics	Ruderal (R)	Combative (C)	Stress-tolerant (S)
Characteristic features	Rapid growth; primary resource capture	Tolerance of specific stresses (e.g., temperature extremes, low water availability, extremely low or high pH, allelopathic chemicals)	Antagonistic ability against competitors
Growth rate	Rapid spore germination and growth	Sometimes slow	Not particularly slow
Enzymatic ability	Relatively narrow ability	Wide ability	Wide ability
Substrates utilized	Easily assimilable	More recalcitrant	More recalcitrant
Timing of reproduction	Early in life cycle	Later in life cycle, sometimes sporadic	Later in life cycle, sometimes sporadic
Commitment of biomass to reproduction	Rapid and substantial	Relatively low	Relatively low
Persistence within the resource	Low; easily replaced	Persistent while specific stress remains	Persistence depends on ability to capture and defend territory

Hyphodontia paradoxa, *Piptoporus quercinus*, and *Trechispora* spp., allowing them to survive severe abiotic stress, e.g., desiccation (see 5 and references therein).

Though the inoculum potential of an individual spore is small, it can be considerably increased if genetically identical spores, e.g., conidia, germinate close to one another. Germ tubes home in on each other and fuse to form a network (14). In contrast, when spores are genetically different, and hence somatically incompatible, competition is likely to result (15). When basidiospores germinate, the mycelia that develop are usually homokaryotic. It is generally thought that this homokaryotic stage is short-lived and that mycelia soon (within hours or days of germination) encounter a suitable conspecific and, following a successful mating, become heterokaryotic. However, even common fungi such as *Trametes versicolor* and *Heterobasidion annosum* can sometimes remain homokaryotic for several years (11, 15–17), and rare species, e.g., *Hericium* spp., might be expected to remain homokaryotic for much longer (18). Homokaryotic and heterokaryotic mycelia do not necessarily behave the same in terms of growth rate, decay ability, competitive ability, etc., though there does not seem to be a general trend where one is better (has higher "fitness") than the other (19 and references therein).

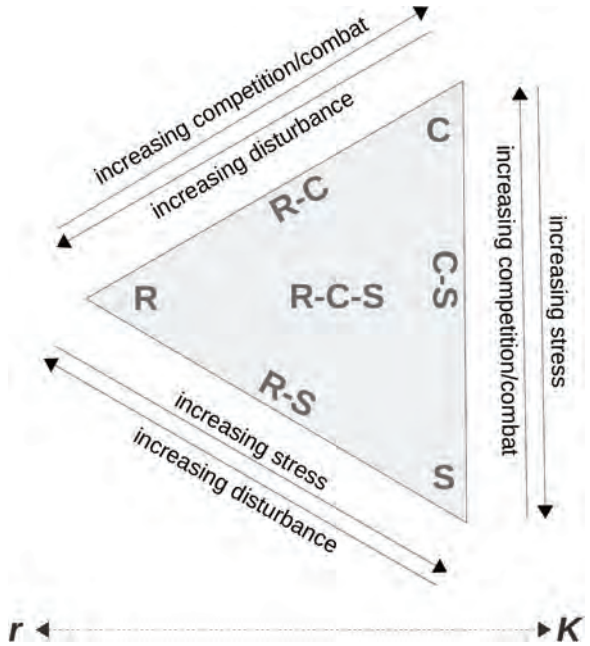


Figure 1 How R-C-S characteristics relate to r-K strategies.

In contrast to arrival as spores, arriving as mycelium allows the fungus to draw upon a much larger supply of nutrients (20). Mycelial spread can be as individual hyphae, albeit sometimes forming dense mycelia or fronts, or as hyphae aggregated to form linear organs—mycelial cords and rhizomorphs (2, 21, 22). Some leaf litter decay fungi can form large patches, e.g., *Collybia* spp. and *Marasmius* spp., or "fairy rings," e.g., *Clitocybe nebularis* (20, 23). These patch formers colonize individual fallen leaves but spread by mycelial growth from one leaf to another as if the litter layer was one large continuous resource. In contrast to mycelial patches, which exhibit no particular pattern, fairy rings comprise a 30- to 40-cm-wide annulus of mycelium which is highly polar, growing outward from an initial site of establishment, with death of older mycelium forming a central zone devoid of the fairy ring former (20, 23).

The mycelial systems of fungi that produce linear organs of aggregated hyphae are unique. The structure of the linear organs covers a spectrum of complexity, from simple loose aggregations through to hyphae that

are highly aggregated to form cords; e.g., *Hypholoma fasciculare*, *Megacollybia platyphylla*, *Phallus impudicus*, and *Phanerochaete velutina* form cords with a thick outer rind that are differentiated internally, in contrast to the thick-walled melanized rhizomorphs of *Armillaria* spp. (21, 22, 24, 25). Rhizomorphs grow from the tip, whereas cords tend to form behind an actively growing front of individual hyphae. When mycelia grow out from a resource, they exhibit different branching patterns which vary between species and depend on many biotic and abiotic factors including size, quality and states of decay of the resource, soil type, microclimatic environment, antagonistic fungi and other microbes, and grazing invertebrates (12, 20, 26, 27). Some can be considered short-range foragers, with highly dense hyphae and mycelia, yielding a mass fractal dimension close to 2 in two dimensions, e.g., *H. fasciculare* (27) (Fig. 2A). They are likely to be successful in encountering small organic food resources. Others are longer-range foragers with more open systems and a lower mycelial mass fractal dimension, e.g., *P. velutina* and *Resinicium bicolor* (Fig. 2B,C). Even longer-range foragers have a mass fractal dimension of close to 1, e.g., rhizomorphs of *Armillaria* spp. (27).

When mycelia of cord-forming species encounter new resources, they are able to exert considerable "inoculum potential" for colonization, being able to draw on nutrient reserves from the mycelial network, which gives them considerable advantage over spores. If colonization of the newly encountered resource is successful, there is often reallocation of mycelial biomass, with thickening of cords interconnecting the original and new resource, and regression of nonconnecting mycelium (2). Thus, a network of cords interconnecting woody resources develops on the forest floor and can operate a sit-and-wait strategy, colonizing wood when it falls onto the network, as well as an active-search strategy (12). These networks can be extensive, though dynamic, covering many square meters or even hectares; networks of rhizomorphs of *Armillaria* spp. constitute one of the largest organisms on the planet (20, 28–32). While in temperate and boreal forests such mycelial networks are confined to the forest floor, in tropical forests similar networks are found both on the floor and in the canopy (33), in the latter case catching small leaf litter and wood components before they reach the forest floor.

Entry and Establishment

Plant tissues that have recently died are usually colonized initially by endophytic fungi already present and/or by prolifically sporing R-selected species which happened to arrive first (34, 35); thus, at early stages the absence of species commonly considered to be later colonizers reflects arrival time rather than an inability to colonize newly available plant tissues. On the forest floor, arrival by mycelium can, however, sometimes happen early on if the resource is located on or close to an active mycelia patch or network of mycelial cords.

Entry and establishment in an uncolonized dead organic resource, be it following arrival as spores or mycelium, depend largely on the enzymatic ability of the fungus. If the fungus has the enzymatic capacity to use compounds available within the resource, it will colonize, establish, and remain present until it (i) is ousted by another species, (ii) is inhibited or killed by adverse abiotic conditions, (iii) uses up the food supply, or (iv) is triggered to fruit or grow out of the resource

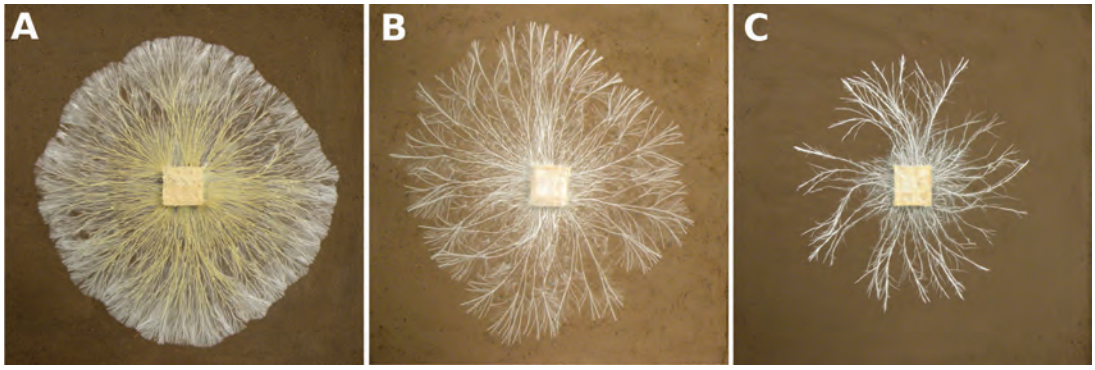


Figure 2 Foraging strategies of cord-forming basidiomycetes growing out of precolonized beech wood blocks across compacted soil. (**A**) *Hypholoma fasciculare*, a short-range forager, produces highly dense hyphae and mycelia. (**B**) *Phanerochaete velutina* is a longer-range forager with a more open cord system. (**C**) *Resinicium bicolor* has an even more open system than *P. velutina*, with thicker cords.

in search of others. If the fungus exits the resource by fruiting, it may or may not commit all of its mycelial biomass to reproduction, depending on its life strategy (see above). Timing of production of reproductive structures also depends on life strategy, ruderal (R-selected) species committing themselves rapidly and prolifically to reproduction and others (C- or S-selected) tending to reproduce later in life and not usually committing all of their mycelial biomass to reproduction (Table 1). Fungi that are able to exit by mycelial spread (those with C- and S-selected characteristics) do so at different times following colonization, with the exact timing varying depending on the species, other biotic and abiotic factors, and on the relative size/nutrient status of the resource compared with other resources in the network (2).

During colonization simple compounds are typically used first followed by more recalcitrant cellulose, hemicellulose, and lignin. Colonization of the nonlignified tissues is relatively easy for mycelia, but woody tissues are more challenging. In wood, most rapid spread is usually along vascular tissues; tangential and radial spread necessitates boring through lignocellulose in the cell walls (35). Plant anatomy often results in the characteristic longitudinally extensive decay columns seen in wood (Fig. 3).

Community Development

Communities that develop initially depend on the environmental conditions when the resource becomes available for colonization, ranging between low environmental stress, where fungi with R-selected characteristics dominate initially, to high stress, e.g., due to extreme abiotic variables, where species that dominate have appropriate S-selected characteristics often combined with some R-selected characteristics (3, 5) (Fig. 4). High-stress conditions include heartwood of trees containing allelopathic compounds; extreme temperature of hot deserts and Arctic, Antarctic, and alpine tundra; and the desiccating conditions of tree canopies. Though some of the initial fungal colonizers have the enzymatic ability to completely break down the resource that they are colonizing, most are usually replaced sooner or later by other species when abiotic conditions worsen (stress aggravation) or improve (stress alleviation), when the habitat is disturbed, or when competition/combat (see next section) with other fungi ensues (Fig. 1). Changes to the abiotic conditions occur due to changes in microclimate but are also brought about by fungi altering the physical and chemical environment as they metabolize the resource they are colonizing (see next section). Disturbance occurs when resources are suddenly made available (enrichment



Figure 3 Sectioned beech trunk showing decay columns running longitudinally through the wood. Arrows indicate dark zone lines (pseudosclerotial plates) surrounding different decay columns.

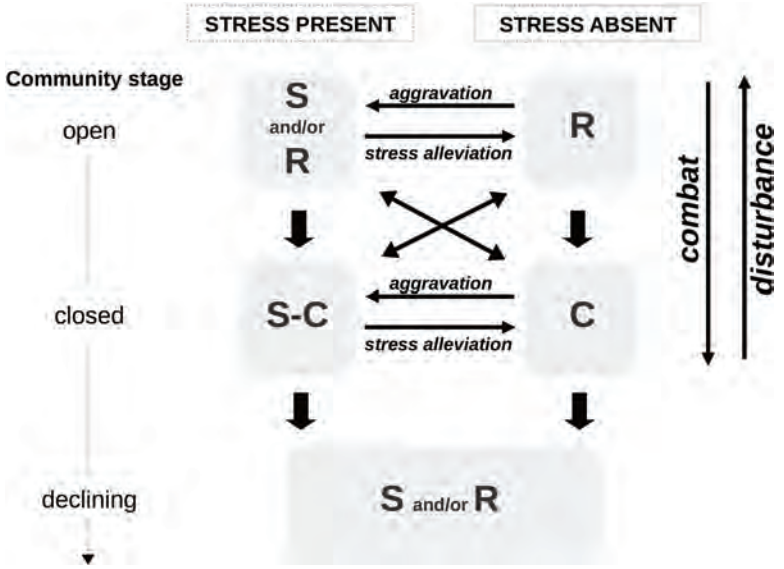


Figure 4 Fungal community development pathways in woody resources. Newly available wood (top) becomes progressively colonized, initially through primary resource capture in an open community stage where there is still unoccupied territory, until all territory becomes occupied, resulting in a closed community where further colonization occurs as secondary resource capture. As the community moves from open to closed, combat becomes the driving force for change. Finally, communities in well-decayed wood are characterized by substrate modification and invasion by soil invertebrates. The ecological characteristics of the dominant organisms are indicated in boxes: R, ruderal; C, combative; S, stress-tolerant. Driving forces are indicated in italic, and direction of community change is indicated by arrows. The community may be driven toward the left by stress aggravation or to the right by stress alleviation, although destructive disturbance will drive the community toward species with R-selected characteristics. (Adapted from references 5 and 58.)

disturbance) or when part or all of the resident mycobiota is destroyed, e.g., following fire. Competition/combat occurs when the expanding territory of fungi in freshly available resources overlaps and when new colonizers arrive at a resource via spores or mycelial spread.

Thus, communities of primary, secondary, and late secondary (or tertiary) colonizers develop. Community development is not a simple ordered sequence, but a complex ever-changing mosaic. The general order of colonizing species—succession—has been determined in many types of organic substrata, but three-dimensional structure has been mostly studied in decaying wood, because the mosaic of different individual fungi is clear to see at all but the earliest and latest stages of community development (Fig. 5A). Since organic substrata are opaque, they have to be destructively sectioned to reveal the individual decay columns. The patterns revealed can be mapped, isolations can be made onto agar media and subsequently identified, or DNA can be directly extracted from wood and the three-dimensional community can be determined (Fig. 3, Fig. 5A). Isolation onto agar has the benefit that isolates of the same species can be paired to determine, in the case of basidiomycetes, whether the isolates are the same genotype/individual based on somatic incompatibility (11, 36). The same can be done with ascomycetes, but different individuals/genotypes can sometimes belong to the same vegetative compatibility group (36, 37), so the situation is not so clear-cut. Experimental pairings can also be made between different species to give indications of relative combative ability, which can aid in understanding of community development pathways.

Sectioning is destructive, so patterns of community development cannot be followed in an individual organic substratum, but rather must be inferred by analyzing many units at different stages of decay. Extracting DNA samples from substrata, e.g., by collecting sawdust from holes drilled into wood, can reveal species composition (e.g., reference 38). Such samples could be extracted at different times to reveal a temporal sequence of colonization, but sampling may alter abiotic conditions in the resource and/or allow different fungi to colonize. The presence of fruit bodies provides a vague idea of the fungi present and has been used to

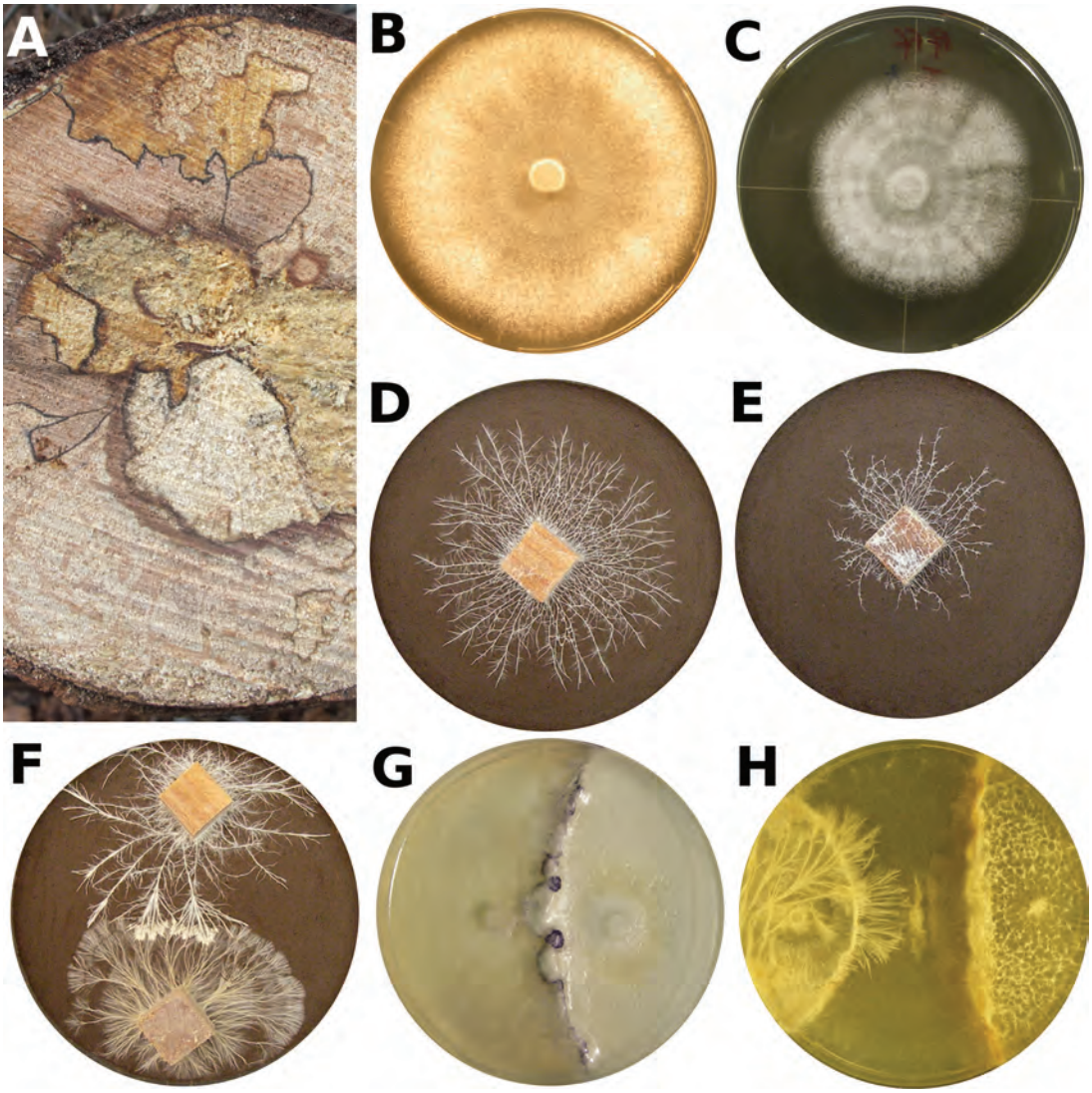


Figure 5 Interspecific interactions of fungi growing in natural and artificial media. (**A**) Cross section of a decaying beech branch with dark zone lines (pseudosclerotial plates) surrounding competing mycelia. (**B, C**) Growth of *Trametes versicolor* when exposed to the diffusible organic compounds (DOCs) from uncolonized malt broth (control) (**B**) or DOCs from *Fomes fomentarius* (**C**). (**D, E**) *Phallus impudicus* cord systems growing across compacted soil when exposed to volatile organic compounds (VOCs) from uncolonized soil (control) (**D**) or VOCs from *Hypholoma fasciculare* growing across soil (**E**). (**F**) Interaction between *H. fasciculare* and *Resinicium bicolor* cord systems across compacted soil. (**G**) Accumulation of reactive oxygen species (ROS) at the interaction zone between *Bjerkandera adusta* (left) and *T. versicolor* (left) on 2% malt agar (MA). ROS are stained purple using nitro blue tetrazolium (methods in reference 92). (**H**) Three-way interaction between mycelia of *H. fasciculare* (left), *P. velutina* (center), and *Stereum hirsutum* (right) growing on 2% malt agar (MA). *H. fasciculare* cords are beginning to encroach over the *P. velutina* mycelium, while *H. fasciculare* itself is overgrown by *P. velutina* cords. A thick barrage separates the mycelia of *S. hirsutum* and *P. velutina*, with a distinct orange/yellow band of pigment deposited in the agar at the regions of contact between the two mycelia.

infer colonization sequence. Sequences of fruit bodies occurring on dung provide an early cautionary tale (see reference 39). The order in which fruit bodies appear and disappear is largely related to their simplicity: mucorales, e.g., *Mucor*, *Pilaria*, and *Pilobolus* spp., are usually visible within a few days, declining after a week; after 5 to 6 days fruit bodies of discomycetes, e.g., *Ascobolus* and *Coproboia* spp., are evident; these are joined fruiting after 9 to 10 days by pyrenomycetes and loculoascomycetes, e.g. *Sordaria* and *Podospora* spp.; finally, basidiomycetes, e.g., *Coprinus*, *Stropharia*, and *Paneolus* spp., fruit. The fungi are, however, often already present in the dung when it is deposited or have colonized very early. Some species have evolved adaptations to passage through the gut and may even be able to germinate and grow while still within the near-anaerobic conditions of herbivore rumens. *Sporormiella minima*, for example, begins to grow before sheep dung is deposited, allowing it to colonize and fruit much more quickly than other species with similarly complex fruit bodies (40).

In wood, the order of fruit body appearance depends, to some extent, on the order within succession, but also on the ecological strategy, with fungi with S- and C-selected characteristics tending to fruit sporadically and much later in their life cycles. However, fruit bodies of some species repeatedly and sometimes almost exclusively follow those of other specific species (41–44). For example, *Antrodiella hoehnelii* almost always fruits following *Inonotus nodulosus* and *Inonotus radiatus*, while *Hericium coralloides* fruits following *Inonotus obliquus, Inonotus cuticularis*, or *Fomes fomentarius* on angiosperm wood in central Europe and Scandinavia (41, 43). This has led to the idea of predecessor-successor relationships and priority effects.

The order in which species arrive at a resource, i.e., the assembly history, affects the composition and development of the community which follows. When earlier colonizing species affect the colonization success of species that arrive later, whether as spores or mycelium, they are described as exerting priority effects (45, 46). Such effects can be stimulatory or inhibitory. Wood decay fungi again provide good examples: they change the resource they occupy both chemically and physically by utilizing different components of the wood cell wall, making nutrients available and altering wood chemistry, pH, and water content. This can prevent some species from capturing territory, acting as a sort of constitutive defense (35), or can select for certain species that prefer the altered environment (44, 47–50). The presence of a certain species can make a resource easier to colonize by specific fungi; for example, wood colonized by *Trametes* spp. may be more attractive to *Lenzites betulina* than is other wood, since the latter is temporarily mycoparasitic (see next section) on the former and can gain easy access via the mycelium of its host (51). Once *L. betulina* has taken over the territory occupied by *Trametes* spp., it switches strategy and exhibits interference competition to defend and gain territory from other fungi (see next section for mechanisms involved in interference competition). Similarly, *Trametes gibbosa* is temporarily mycoparasitic on *Bjerkandera adusta* (51). Priority effects are common in the development of wood decay communities (e.g., references 45, 46, 49, 52–55), although they may be less evident in the later stages of decay (56).

COMPETITION

Competition is the negative effect that one organism has on another by using up, or inhibiting access to, a resource of limited availability (57). When one organism inhibits the other and limits access to resources, it is termed interference competition, whereas when one organism depletes a resource, consequently reducing its availability, it is termed exploitation competition (57). The sequestration of nutrients by mycelia growing through soil, hence preventing other fungi from using them, is an example of exploitation competition. However, when saprotrophic basidiomycetes and xylariacious ascomycetes are growing in and feeding on solid organic resources, e.g., wood and leaf litter, the distinction between exploitation competition and interference competition is not clear, and they cannot sensibly be divorced from each other (58). This is because these fungi compete to obtain and defend three-dimensional territory within the organic resource; within the territory the resources can be used at the fungus' leisure. Thus, competition for nutrients is effectively brought about by competition for territory/space.

Fungal competition in organic resources is often divided into (i) primary resource capture, when a fungus colonizes and gains influence over a previously unoccupied territory/resource, and (ii) secondary resource capture, when a fungus captures territory from fungi that have already colonized a resource (3, 58). Another aspect to secondary resource capture is defense of territory from potential invaders. R-selected characteristics favor primary resource capture, whereas success in secondary resource capture depends on combative/antagonistic mechanisms (predominantly C-selected characteristics). Combative/antagonistic interactions can occur at a distance and following contact, comprising mycoparasitism and larger-scale mycelial interactions.

Antagonism at a Distance

Antagonism between fungi can occur in the absence of mycelial contact, through the production of volatile and diffusible organic compounds (VOCs and DOCs, respectively) (59). Fungi produce a wide range of these so-called secondary metabolites, spanning a variety of chemical classes, from short-chain alcohols and ketones to aromatic compounds and terpenes (60–62). Different species tend to produce a characteristic metabolite profile (63, 64), although this profile can be perturbed by growth substrate, pH, culture age, and temperature (65–67).

While DOCs have antagonistic potential in circumstances in which they can accumulate or diffuse through substrata (i.e., locally), VOCs function in much more heterogeneous environments and can act over greater distances (68, 69). Mycelia exposed to the DOCs or VOCs of a competitor exhibit altered spore germination, mycelial morphology, foraging behavior, and enzyme production (47, 67, 68, 70–72). For example, the extension rate of *T. versicolor* was reduced when grown on media containing DOCs from *F. fomentarius* cultures (Fig. 5B and C), and the extension of *P. impudicus* cords across soil was reduced as a result of exposure to VOCs from *H. fasciculare* (Fig. 5D and E) (67). The antagonistic potential of VOC and DOC profiles depends on the chemical composition of that profile and the susceptibility of the combatants; effects of VOCs and DOCs may be stimulatory and function as attractants to competitors, mycoparasites, or invertebrates (47, 72).

Mycoparasitism

Mycoparasitic relationships occur when one mycelium gains nutrition directly from another (35). The mycoparasite may cause the death of the host mycelium and utilize nutrients from the dead or dying hyphae (necrotrophy), or it may derive nutrition from living mycelia (biotrophy). There is a spectrum of relationships between these extremes, and some fungi may grow biotrophically on certain hosts but necrotrophically on others (73). Not only do fungi parasitize other mycelia, but they can also parasitize fruiting bodies, spores, and sclerotia (73).

Biotrophic mycoparasitism

Biotrophic mycoparasitic relationships are complex, controlled, and specialized associations between mycoparasite and host. Biotrophic mycoparasites have a narrow host range, and the mycoparasite is frequently dependent on the host for survival; for example, *Coniothyrium minitans* is an obligate mycoparasite of certain *Sclerotinia* and *Botrytis* spp. and lacks a free-living saprotrophic stage (74). The establishment of biotrophic associations requires high specificity in recognition between the host and the mycoparasite (73). The associations are relatively nondestructive, with the cytoplasm of the host remaining relatively healthy, but abstraction of nutrients from the host results in reduction in host biomass, often causes distortion of host hyphae, and has adverse effects on host sporulation (73, 75, 76). Three subdivisions of biotrophic mycoparasitism have been described based on physiological characteristics. First, the intracellular biotrophs function by the entire thallus entering and developing within the host cells and absorbing nutrients directly from the host cytoplasm. Second, haustorial biotrophs penetrate host cell walls by the production of appressoria and the development of specialized absorptive branches (haustoria) which invaginate the host plasma membrane. Host nutrients are absorbed across the plasma membrane into the haustorium. Third, fusion or contact biotrophs produce specialized hyphae which closely adpress to the host hyphae and form channels or micropores in the host cell wall, allowing the biotroph plasmalemma to fuse with that of the host and absorb nutrients directly from the host cytoplasm (37, 76).

Necrotrophic mycoparasitism

Necrotrophic mycoparasites tend to have a broad host range and utilize relatively unspecialized, destructive parasitic mechanisms. For many necrotrophs parasitism is more opportunistic than biotrophy and, as mentioned above, can even be temporary, providing the parasite with a means of access to a different food source. As with biotrophic mycoparasites, the necrotrophs can be subdivided based on their physiological relationship with the host. Noninvasive necrotrophs make contact with, or grow very close to (within a few micrometers), host hyphae, which they attack by a process known as "hyphal interference." The mycoparasite secretes nonenzymic diffusible toxins, which cause impaired membrane function and result in lysis of organelles, invagination of the plasma membrane, and eventual death of the hyphal compartment (73). Death of the whole mycelium may occur if multiple contacts are made. In contrast, invasive necrotrophs coil around and penetrate host hyphae (73). Contact and recognition of a host often stimulates production of specialized structures on the mycoparasite cell wall, with which it binds to host hyphae (77). The mycoparasite produces antifungal metabolites and lytic enzymes to disrupt host cytoplasm, resulting in vacuolation and lysis of hyphal walls and organelles. For example, vigorous

necrotrophs in the genus *Trichoderma* secrete antibiotic peptides called peptaibols, which disrupt cytoplasmic membranes and cause hyphal leakage and eventual cell death, and they also secrete cell wall-degrading chitinases and proteases (78).

Larger-Scale Mycelia Interactions: Antagonistic Mechanisms

For saprotrophic fungi, the territory occupied by a mycelium is also its nutrient source, and as such, mycelia attempt to maximize their territory by replacing other mycelia and defending themselves from replacement. This is clearly seen in communities of wood decay fungi; the territories occupied by different mycelia in decaying wood are often delineated by pigmented barriers, or "pseudosclerotial plates," which are the interfaces between competitors (Fig. 5A) (58). The establishment of physical contact between two competing mycelia, often termed "gross mycelial contact," results in large-scale changes in the growth, gene expression, and metabolite production in both competitors and in the induction of antagonistic mechanisms. The outcomes of antagonistic interactions range from replacement of one competitor by another to deadlock, where neither species can capture any territory from the other (58). Between these extremes are partial replacement, where one species is able to capture some but not all of the opponent's territory, and mutual replacement, where one species obtains some of the territory formerly occupied by the other and, simultaneously, *vice versa*. Outcomes are determined by the relative abilities of the opponents to capture and defend territory, and different species may exhibit different strategies during combat, displaying traits that may benefit them in attack and/or defense (79). Some fungi are good at both attack and defense, whereas others are good at one of these but not the other. For example, the secondary colonizer *Stereum hirsutum* is relatively poor at gaining new territory in decaying beech wood but can defend the territory it occupies against more combative later secondary colonizers such as *H. fasciculare* and *P. velutina* (49). Further, the progress and outcomes of interactions can be altered and even reversed by changing environmental conditions such as invertebrate grazing, gaseous regime, water availability, and temperature (79–82).

Morphological changes

Antagonistic mechanisms utilized by mycelia to attack or defend against competitors include morphological changes, production of enzymes and toxins, detoxification of competitor toxins, and alteration of metabolic rate. Changes in mycelial morphology are most dramatic in areas in direct contact with the competitor—the interaction zone. Hyphae may aggregate to form defensive barrages to physically block invaders or to form invasive replacement fronts or cords to penetrate competitor defenses (Fig. 5F and H) (58). Different types of hyphal assemblage can be found in different regions of the same interaction front, indicating that antagonistic mechanisms are deployed dynamically and in response to local stimuli (68). Changes in morphology during interactions are reflected in changes in expression of genes involved with cell division, cellular transport, and cytoskeleton rearrangement compared to noninteracting mycelia (83–85).

Secondary metabolite production

Profiles of VOCs and DOCs alter quantitatively and qualitatively during interactions, often involving production of interaction-specific compounds not produced by either competitor during growth alone (66–68, 72, 86–88) (Table 2). Interaction-specific VOCs are often identified as terpenoids, frequently sesquiterpenes (66, 67, 87). Many sesquiterpenes are known to be bioactive, displaying antifungal activity or functioning as attractants or repellants to fungi and invertebrates (61, 89, 90). Some compounds that were produced constitutively may be upregulated following contact with a competitor (Table 2); for example, the production of a potentially antifungal quinolinium-type compound by *H. fasciculare* doubled during interactions with *T. versicolor* compared to during growth alone (72).

Accumulation of reactive oxygen species (ROS)

ROS accumulate at interaction zones, although their exact role is unclear (Fig. 5G) (84, 91, 92). ROS may be produced by one or both competitors to generate a toxic oxidative environment, and increases in potential sources of ROS, such as increased expression of genes encoding NADPH oxidase and increases in peroxidase and phenoloxidase activity, have been detected at interaction zones (84, 92, 93). Increases in expression of genes encoding catalase and putative DNA repair proteins have also been detected at interaction zones, which suggests attempts by the mycelium to mitigate ROS toxicity and repair oxidative damage (83, 84). However, a direct role for ROS toxicity during interactions between wood decayers seems unlikely since these fungi are adapted to tolerate the oxidative stress caused by activity of their own ligninolytic enzymes. Instead, ROS accumulation may be incidental and occur as a result of disruption of cellular metabolism caused by

Table 2 Secondary metabolite production during antagonistic interactions[a]

Chemical class	Compound name	VOC/DOC	Change in production	Interaction (species) reported in and substrate	Reference
Benzenoid	1,2-Dihydroxyanthaquinone	DOC	Increase	*Stereum hirsutum* vs. *Coprinus disseminatus* on MA	86
	5-Methyl,1,3-cyclohexadiene	VOC	Interaction-specific	*Trametes versicolor* vs. *Stereum gausapatum* in malt broth	72
Carboxylic acid	Fusaric acid	DOC	Increase	*Ustilago maydis* vs. *Fusarium verticillioides* on PDA	103
	Malic acid	DOC	Increase	*S. hirsutum* vs. *C. disseminatus* on MA; *Trichoderma viride* vs. *Schizophyllum commune* on PDA	86, 100
Sesquiterpene	α-Bulnesene	VOC	Increase and decrease (depending on species involved)	*Hypholoma fasciculare* vs. *Phanerochaete velutina*; *Resinicium bicolor* vs. *Phallus impudicus*; *Phanerochaete veutina* vs. *P. impudicus*; *R. bicolor* vs. *P. velutina*; *H. fasciculare* vs. *P. impudicus*; all in beech wood	67
	Selinene (α and β)	DOC	Interaction-specific	*Nodulisporium* sp. vs. *Pythium aphanidermatum*; *Nodulisporium* sp. intraspecific interaction; both on PDA	88
Monoterpene	Pinene	VOC	Interaction-specific	*T. viride* vs. *Aspergillus niger* in straw powder	87
	γ-Terpinene	DOC	Interaction-specific	*Nodulisporium* sp. vs. *P. aphanidermatum* on PDA	88
Sugar alcohol	Erythritol, meso-erythritol	DOC	Increase	*T. viride* vs. *S. commune* on PDA; *S. hirsutum* vs. *Coprinus micaceus* on MA; *S. hirsutum* vs. *Coprinus disseminatus* on MA	86, 100
Sugar alcohol	Glycerol	DOC	Decrease	*T. viride* vs. *S. commune* on PDA	100
Ketone	3-Octanone	VOC	Increase and decrease (depending on species involved)	*H. fasciculare* vs. *P. velutina*; *R. bicolor* vs. *P. impudicus*; *P. veutina* vs. *P. impudicus*; *R. bicolor* vs. *H. fasciculare*; *H. fasciculare* vs. *P. impudicus*; all in beech wood	67
	Bicyclo-oct-6-en-3-one	DOC	Interaction-specific	*Nodulisporium* sp. intraspecific interaction on PDA	88
Alkane	Alkanes (C7-C54)	VOC/DOC	Interaction-specific	*T. viride* vs. *A. niger* in straw powder; *Nodulisporium* interspecific interaction on PDA	87, 88
Alcohol	2-Methyl-1-butanol	DOC	Interaction-specific	*Nodulisporium* sp. vs. *P. aphanidermatum* on PDA	88
Aldehyde	2,3,4-Trihydroxybutanal	DOC	Increase	*T. viride* vs. *S. commune* on PDA	100

[a]Select examples of the volatile and diffusible organic compounds (VOCs and DOCs, respectively) that have been detected as changing in production during interspecific fungal interactions. Metabolites belong to a wide variety of chemical classes and may increase or decrease in production during interactions relative to solo growth or may be specific to the interaction. Interaction experiments were performed in a variety of substrates, including malt agar (MA) or broth, potato dextrose agar (PDA), beech wood, and straw powder.

other antagonistic mechanisms. Alternatively, increases in ROS levels may function as a defense signaling response similar to that in plants, for example, triggering biosynthesis of pigment (92, 94). Increases in another potential signaling compound, nitric oxide, have also been detected during interactions between *Phellinus morii* and *I. obliquus*, triggering the production of antifungal phenylpropanoid metabolites (95).

Oxidative enzyme activity

Activities of peroxidases and phenoloxidases (laccases) are also upregulated at interaction zones (19, 83, 93)

(Table 3). This may function to increase decomposition and could be associated with increased utilization of the resource during combat. However, laccases and peroxidases are also secreted in response to stress and could function during interactions to detoxify competitor VOCs and DOCs (19, 85). Other enzymes involved in detoxification are also upregulated during interactions; for example, increases in the expression of genes encoding oxidoreductases, aldo/ketoreductases, and glutathione *S*-transferases were detected in *T. versicolor* and *Pycnoporus coccineus* during interactions with various competitors (84, 85). Laccases may also function to wall off and protect hyphae during interactions through production of melanin, which insulates hyphae from ROS, toxins, temperature extremes, and hydrolytic enzymes and may also have direct antibiotic properties (96, 97). Pigmentation is frequently observed at interaction zones (Fig. 5H), and while there is some indication that this is the result of deposition of DOCs, this may also be the result of melanization (72).

Table 3 Extracellular enzyme production during antagonistic interactions[a]

Enzyme	Function	Proposed role in interactions	Change in activity	Interaction (species) reported in and substrate	Reference
Laccase (phenoloxidase)	Degradation of lignin	Detoxification of competitor metabolites; pigment production; ROS generation	Increase	e.g., *Trametes versicolor* vs. *Stereum gausapatum* on MA; *Humicola grisea* vs. *Trichoderma harzianum* in CLN broth; *Pleurotus* sp. vs. *Dichomitus squalens* on wheat straw	19, 93, 104
			Decrease	e.g., *T. versicolor* vs. *Fomes fomentarius* on MA	19
Peroxidase					
Manganese peroxidase	Degradation of lignin	Detoxification of competitor metabolites; pigment production; ROS generation; enhanced nutrient uptake	Increase	e.g., *Trametes maxima* vs. *Paecilomyces carneus* on PDA; *Pleurotus ostreatus* vs. *Phanerochaete chrysosporium* in NWS; *T. versicolor* vs. *S. gausapatum* on MA	19, 105, 106
Lignin peroxidase				e.g., *Pleurotus ostreatus* vs. *Phanerochaete chrysosporium* in NWS	105
General peroxidase				e.g., *Phlebia radiata* vs. *Phlebia rufa* on MA; *Serpula lacrymans* vs. *Coniophora puteana* on MA	107, 108
Cellulase	Cellulose degradation	Enhanced nutrient uptake	Increase		
β-Glucosidase				e.g., *T. versicolor* vs. *Bjerkandera adusta* on MA	19
α-Glucosidase				e.g., *H. fasciculare* vs. *Phanerochaete velutina* on soil	98
Cellobiohydralase				e.g., *H. fasciculare* vs. *P. velutina* on soil	98
Cellobiase				e.g., *T. versicolor* vs. *T. harzianum* in LN broth	99
N-acetyl glucosaminidase (chitinase)	Chitin degradation	Attack of competitor cell walls, degradation after secondary colonization	Increase	e.g., *T. versicolor* vs. *Hypholoma fasciculare* on MA; *Fomitopsis pinicola* vs. *Resinicium bicolor* on spruce veneer	19
Acid phosphatase	Phosphate release	Enhanced nutrient uptake	Increase	e.g., *T. versicolor* vs. *Daldinia concentrica* on MA; *H. fasciculare* vs. *P. velutina* on soil	19, 98

[a]Select examples of enzymes with changes in activity detected during interspecific fungal interactions relative to growth in solo cultures. Interactions were performed in a variety of substrates, including cellulose low nutrient (CLN) broth, malt agar (MA), potato dextrose agar (PDA), wheat straw, wheat bran-neem hull-sugarcane bagasse (WNS), low nitrogen (LN) broth, spruce veneer, or across soil. The interactions reported represent a few examples, and only a fraction of the studies that have been performed are included in the table.

Energy expenditure during interactions

Antagonism is energetically expensive. Production of invasive mycelial cords by one or both competitors is associated with increases in respiration, indicating that this requires upregulation of metabolic processes (49). Enhancement of nutrient acquisition through increased production of cellulases and phosphatases occurs at interaction zones and throughout the competing mycelia (85, 98, 99). The concurrent reduction in biomass accumulation during interactions between *P. coccineus* and *Coniophora puteana* suggests that this increased nutrient acquisition functions to fund antagonistic mechanisms rather than mycelial growth (85). Metabolism was also found to increase in newly captured territories (i.e., regions where a mycelium had replaced a competitor), and it is likely that the observed increases in activity and gene expression of proteases and chitinases in these regions function to recycle the mycelium of the displaced competitor (85, 100–102). Similarly, genes whose products are involved in carbohydrate and nitrogen metabolism were upregulated in *T. versicolor* mycelium during interactions in which it replaced competitors, but not during interactions in which it was out-competed (84).

CONCLUSIONS

Fungal community development within decaying resources is ultimately driven by the abiotic conditions the resource is subjected to and by the local pool of potential colonizing species. Fungi have evolved different life history strategies to exploit different niches during community development within decaying resources, although certain species may often have combinations of characteristics from different strategies or vary in their strategy during different stages of the life cycle or in different contexts. The communities that develop in newly available resources depend on the levels of abiotic stress present; this determines which of the latently present colonizers, and which of those that arrive as spores or via mycelial spread from local species pools, are most likely to establish within the resource. Changing abiotic conditions, modification of the resource by colonizers, and arrival of more combative species drive shifts in community composition, and in some cases the assembly history determines subsequent community development. Acquisition of previously colonized resources by more combative species is achieved through the deployment of antagonistic mechanisms, which can begin before mycelia establish physical contact through production of volatile and diffusible organic compounds. Interacting mycelia undergo a slew of morphological and biochemical changes, which may be aggressive or defensive in function, and the changes that occur differ depending on the combination of species involved. Interaction outcomes, and thus community change, are determined by the relative combative abilities of the fungi involved, but these outcomes can be altered or even reversed under different environmental conditions.

Many questions remain to be answered for us to fully understand the processes underlying community development of saprotrophic fungi. First, how far do spores spread, and how do they manage to establish within resources that are already colonized; what exactly is the success rate of a spore? Perhaps communities within decaying resources are determined through all initial stages of decay by propagules that are latently present; emerging sequencing technologies will allow a much more comprehensive profile of latent colonizers to assess the extent of their contribution to community development. Further, how strong are priority effects and how resilient are these pathways of community development to global environmental change? Finally, utilization of emerging molecular and biochemical approaches will allow better understanding of the mechanisms involved in antagonism or facilitation within decay communities, which drive changes in community composition throughout the decomposition process.

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Long-Distance Dispersal of Fungi

14

Jacob J. Golan and Anne Pringle

INTRODUCTION

The relative degree to which organisms move is a process operating at multiple temporal and physical scales (1). In recent years dispersal has received a great deal of attention in fields ranging from mathematics and physics to ecology and molecular biology, but only a patchy framework exists to explain dispersal over very large distances. Modeling patterns of long-distance dispersal (LDD) among macroorganisms, ranging from vertebrates and flying insects to seed plants, appears tractable, but documenting the geographic distributions and dispersal dynamics of microscopic propagules and microbes presents multiple theoretical and methodological challenges (2–4). The majority of empirical research directly measuring the dispersal of microbes or microscopic propagules is restricted to relatively short distances, and tracking dispersal at greater spatial scales involves mathematical or genetic models, e.g., in studies of moss (5–9), ferns (10–13), bacteria (14–19), and fungi (19–23). However, fitting dispersal data (e.g., from the tracking of spore movement) to mathematical functions often over- or underestimates LDD and imprecisely describes the trajectory of spore movement across large distances (24–28). Inferences based on population genetics data capture rare instances of successful LDD but incompletely describe underlying demographic processes and typically cannot speak to mechanisms of LDD (1). Besides the limitations of mathematical and genetic methods, important details about the natural history of species are often ignored or remain unknown, leaving many questions unanswered, including, e.g., how ephemeral propagules remain viable while exposed to harsh environments over extended periods of time.

Here we consider LDD as it relates to fungi. Although most research focuses on only a small number of fungi, the kingdom is extremely diverse, housing an estimated 1.5 to 10 million species (29). The ability of fungal dispersal structures (e.g., conidia, basidiospores, ascospores, sclerotia, etc.) to disperse over large distances may be highly context dependent (Fig. 1). Moreover, while LDD for a rust fungus, e.g., *Puccinia graminis*, may be over several kilometers, LDD for a bird's nest fungus, e.g, *Crucibulum laeve*, may be only several dozen meters (30, 31). The delimitation of cryptic species by phylogenetic techniques has also in many cases revealed that a fungus once considered widespread in fact consists of several separate species, each with nearly indistinguishable morphological characteristics, and raises questions about the prevalence of LDD (32–34). Further complicating matters, direct evidence for LDD beyond several kilometers is lacking (35). Thus, any discussion of the dispersal dynamics of fungi as a whole is problematic, especially if comparisons are made or inferred between one fungal group and another (e.g., aquatic fungi compared to ectomycorrhizae) (19, 22, 23, 30, 36).

One common feature among sporulating fungi is the tremendous abundance of both sexual and asexual spores (e.g., a single gall of *Ustilago maydis* [corn smut] contains up to 25 billion spores, and a single sporangium of *Rhizopus stolonifer* [common bread mold], up to 50,000) (35). Fungal spores are orders of magnitude smaller than the smallest seeds—smaller than most moss and fern spores and comparable in size to some plant pollen (e.g., *Triticum aestivum*, or wheat, pollen) (37–40) (see Fig. 2 and 5). However, unlike pollen, many fungal spores are short-lived and highly susceptible to desiccation and UV radiation, and it is often unclear whether spores survive, e.g., transcontinental and oceanic transport (41–45).

Given these taxonomic, empirical, and methodological challenges, a sound conceptual framework to guide and synthesize research is urgently needed. Mycologists have yet to integrate the abundant physiological, morphological, and biogeographic data available for hundreds of species into a unifying paradigm of fungal

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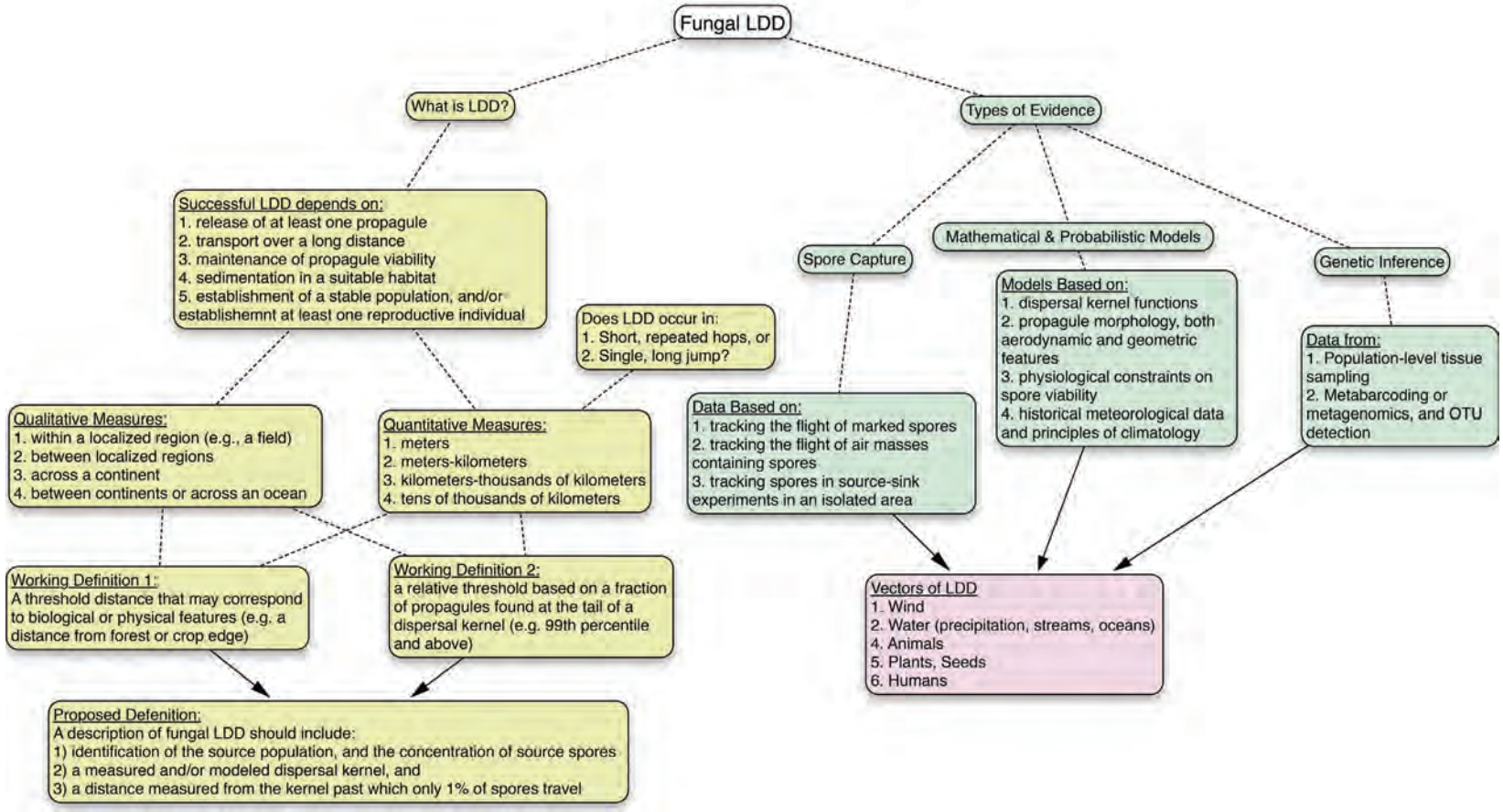


Figure 1 A framework for understanding fungal LDD.

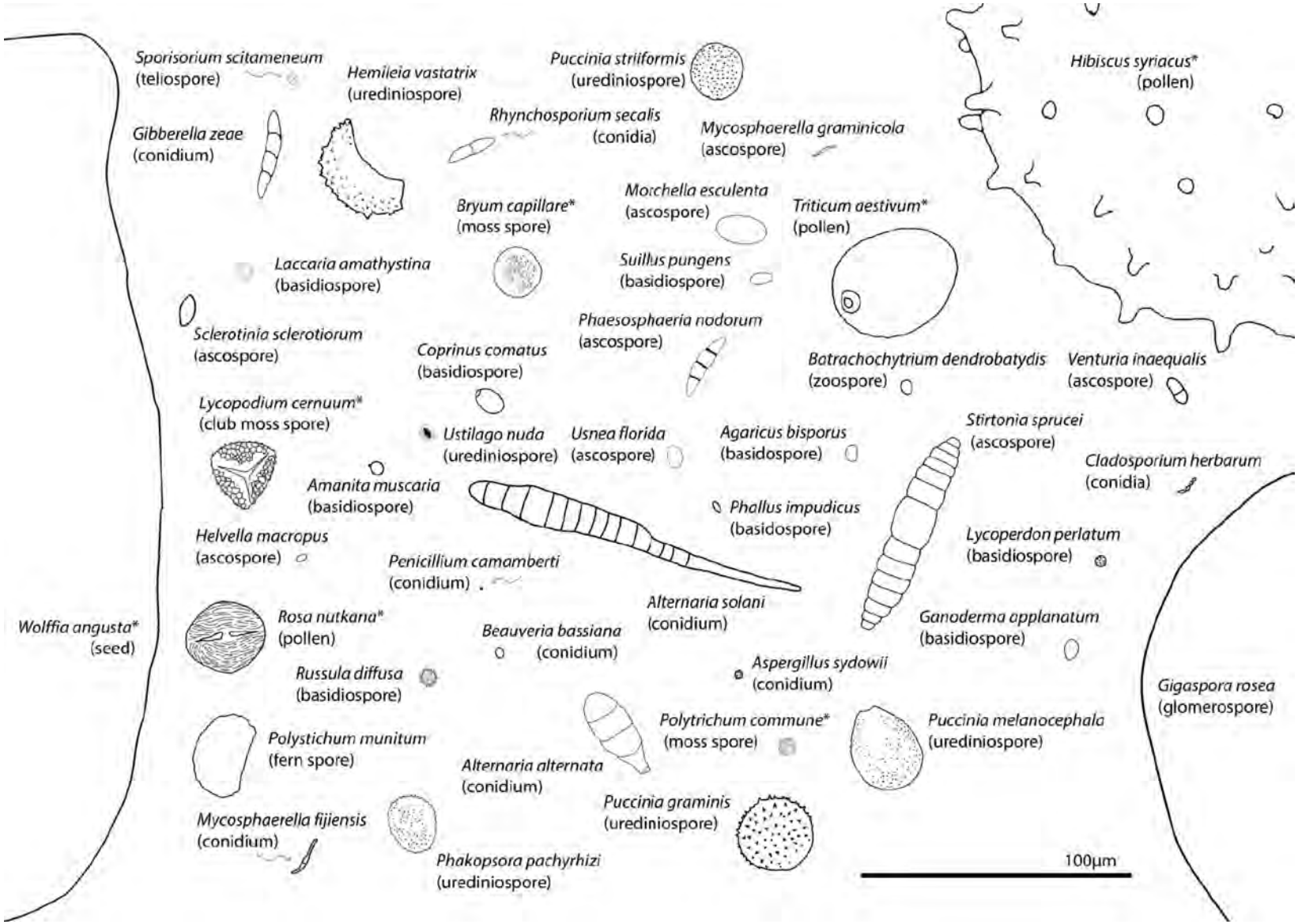


Figure 2 Sizes of fungal spores and other airborne particles. Some species are wind dispersed (e.g., *P. graminis*), while others have other means of dispersal (e.g., *Gigaspora rosea*). The smallest plant seed, *Wolffia angusta*, the pollen grains of *Hibiscus syriacus* and *T. aestivum*, and a glomerospore of the arbuscular mycorrhizal *Gigaspora rosea* are provided for comparison. Species labeled with an asterisk are not fungi.

LDD. If comparisons are to be effective or relevant, the highly relative nature of the spatial scales involved must be explicitly acknowledged in any discussion of LDD (46).

DEFINING FUNGAL LDD

In a general framework focused on dispersal, Nathan (28) highlights two general definitions of LDD that are often used in studies of animals and plants: movement exceeding (i) an absolute threshold equivalent to a chosen distance (e.g., 100 km) and (ii) a relative threshold based on a fraction of propagules found at the tail of a dispersal kernel (e.g., 99th percentile and above). However, a translation of these definitions to research on fungi is hindered by the incommensurate priority given to plants and animals in dispersal ecology (cf. 47–49) and by a lack of appropriate empirical data (e.g., spore sources are often inferred through reverse trajectory models that reveal little about source inoculum density, making inferences about fungal dispersal kernels [required by definition ii] difficult) (1, 28, 50). Moreover, definitions involving absolute threshold distances involve discretionary demarcations of LDD, resulting in a lack of consistency among studies. For example, definitions of LDD range from beyond 100 m (*Fusarium graminearum*), to beyond 1,000 m (*Mycosphaerella fijiensis*), to transoceanic transport (*Aspergillus sydowii*) (43, 51, 52). Using definitions based on a relative threshold facilitates comparisons of dispersal kernels of different species, but only if a common percentile is routinely used.

While it may be appropriate to have alternative definitions of fungal LDD for different species, at the moment there is no comprehensive approach to organizing the myriad methods used to think about fungal LDD. An accurate description of successful LDD must include, at a minimum, the magnitude of the source inoculum, the physical and biological probability of LDD (including, e.g., the vector[s] involved and the longevity of spores or tissues), the availability of suitable landing sites, and the probability of establishing a stable population and reproducing (Fig. 1). Any of these variables can prevent successful dispersal, perhaps explaining why fungal LDD appears extremely rare. Additionally, differences between stepwise vs. single-leap LDD must be distinguished. LDD involving sequential, shorter-distance dispersal is likely the more common phenomenon, while LDD involving a single successful spore moving a long distance is a very low-probability event that would coincide with optimal conditions for both fungus and vector.

To unify the disparate approaches used to describe and measure fungal LDD, we propose a synthetic three-part definition built on the framework presented by Nathan (1, 28). Any description of fungal LDD should include (i) identification of the source population and a measure of the concentration of source spores and (ii) a measured and/or modeled dispersal kernel. With this information LDD is defined as (iii) the distance found within the dispersal kernel beyond which only 1% of spores travel (Fig. 3). The 1% threshold provides a useful, common reference point; other choices are possible, but in any discussion the chosen threshold should be clearly identified. Using this standard definition, or discussing how any particular experiment relates to this definition, would facilitate an integrated approach to understanding fungal dispersal.

MEASURING LDD

Empirical measures of spore dispersal are difficult to make, but direct measures of movement remain critical to understanding the scale of a species' dispersal as a whole (51–53). It cannot be assumed that spores traveling beyond the limits of an experimental setup are statistically and/or ecologically insignificant. Moreover, while spore viability is often ignored, successful LDD requires that, e.g., a spore that has crossed an ocean is also viable. Novel approaches to measuring both spore trajectories and the probabilities of survival are critically needed, and experiments involving creative thinking, and perhaps taking advantage of new technologies, will likely help to better address the many unanswered questions about fungal LDD.

Once a greater array of empirical dispersal data is available, new dispersal kernels can be developed to better quantify fungal LDD (for a review of kernel functions see reference 54). However, many kernel models are best suited to describe either the source or tail end of dispersal, but not both simultaneously, and when applied to entire trajectories, such models tend to either over- or underestimate LDD (24–27). Describing the mathematics behind these models is outside the scope of this review, but examples of their use can be found in many studies of fungi (31, 45, 55–58).

The majority of studies of fungal LDD employ molecular approaches to compare the genetics of populations across a geographic range. However, genetic inferences reveal little about the underlying biological, physiological, and ecological forces at play and may be less relevant to our proposed definition. Studies often compare allele frequencies among discrete populations to infer dispersal, e.g., among Southern Hemisphere

populations of *Ganoderma applanatum-australe*, globally distributed populations of *Tuber* species, and pan-Arctic populations of many ectomycorrhizal fungi (20, 21, 59, 60). If two populations that are very far away from each other appear closely related in a phylogeny (e.g., Israeli populations appear more closely related to populations from Indiana than they are to Syrian populations), then LDD is inferred (Fig. 4) (61–64). Phylogenetic methods allow inferences of rare LDD to be made with less intensive sampling than the direct capture of spores but cannot provide critical information on spore longevity, nor information about the role of meteorological patterns, spore physiology, putative vectors, and human mediation. Genetic approaches cannot be used to model dispersal kernels and reveal little about dispersal mechanisms, regardless of geographic scale.

However, the best examples of LDD are based on a variety of approaches. Considering the different limitations to direct sampling, statistical modeling, and genetic inference, it is not surprising that the best-known cases of fungal LDD are typically generated using these methods in combination. For example, several reports of fungal trans-Atlantic dispersal in Saharan dust manage to capture viable fungal material, describe a dispersal kernel, and use meteorological backtracking to identify air masses as having originated from Africa (42, 43, 65–67). For other taxa, researchers sample directly from within the planetary boundary layer using towers or aircraft, in addition to tracking the trajectories of air masses (68–71). Genetic methods are also combined with spore capture techniques, usually by first determining the genotype of a specific fungal strain and subsequently allowing it to release spores (58). The relative proportions of that genotype collected by spore traps are then used to construct a dispersal kernel.

DISPERSAL VECTORS

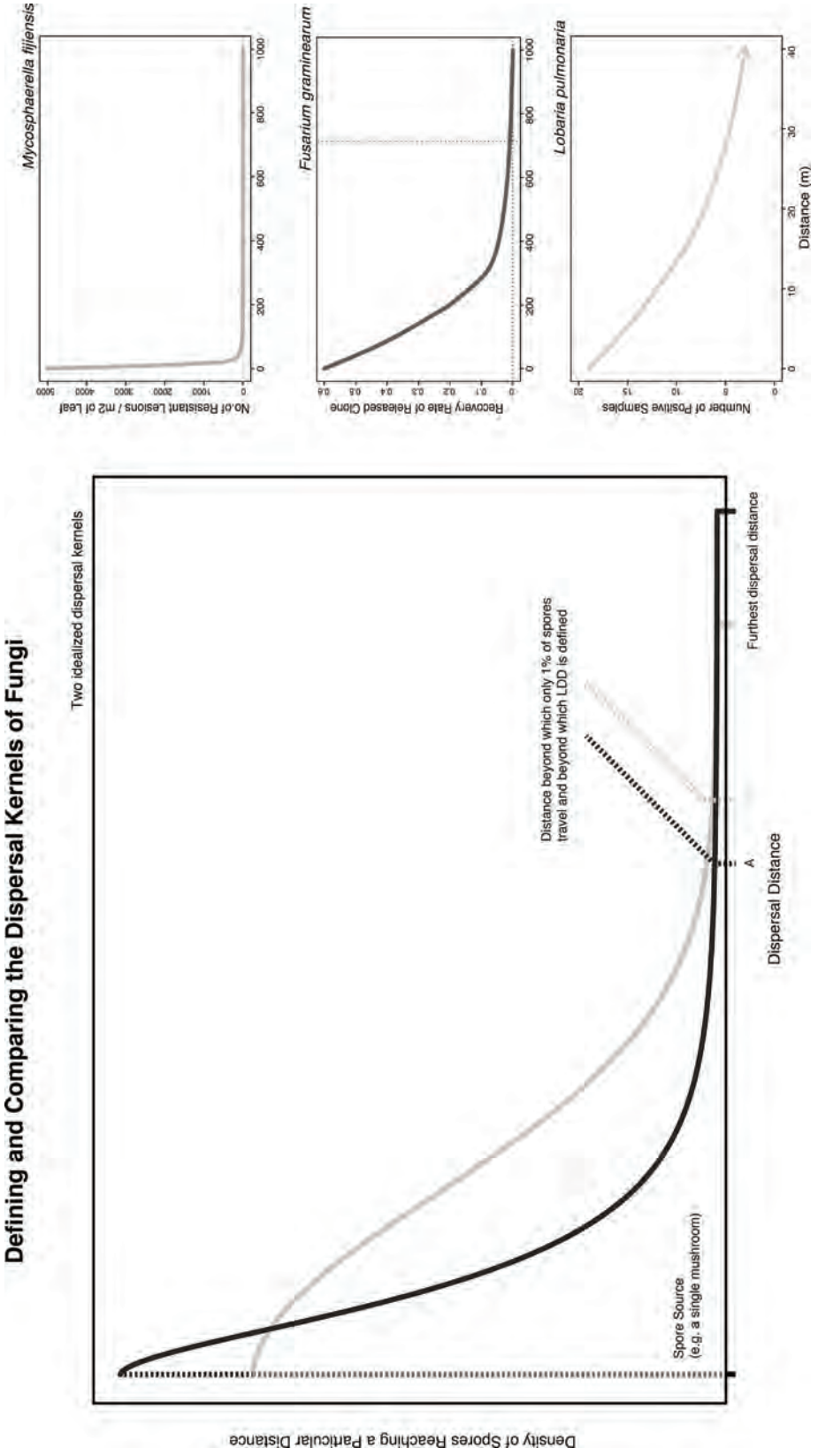
Wind

Wind is the most commonly considered vector of fungal dispersal. Aerosolized course particles (greater than 2.5 μm in diameter) have been photographed moving hundreds of kilometers from northern Africa across the Atlantic, depositing an estimated 500 million to over 1 billion tons of material per year in the Caribbean and Amazon basin (43, 66). Sand, soil, and, in smaller proportions, biological matter including bacteria and fungal spores have also been found in air samples retrieved from towers or aircraft (36, 68, 71). Further evidence that spores can move in the atmosphere is provided by tracking wind patterns. The biogeography of mosses and ferns, as well as lichens, in the Southern Hemisphere may be better described by wind patterns than by geographic distances between land masses (72), providing indirect evidence for LDD via "wind highways" (45). Wind patterns are also used to infer atmospheric LDD of fungal pathogens, e.g., the introduction of *Hemileia vastatrix* (coffee leaf rust) from Africa to Brazil, and of *Puccinia melanocephala* (sugarcane rust) from West Africa to the Caribbean and the United States (73, 74).

Samples of dust from surfaces are also used to infer fungal LDD. Metabarcoding data taken from North American dust reveal a low percentage of common species across regions, but some degree of overlap suggests that LDD is a real, albeit rare, phenomenon (19, 23). Alternative hypotheses posit that the ubiquity of some species is caused by short-distance dispersal over long time scales or that species appearing broadly distributed are complexes of cryptic species, each with restricted geographic ranges (75, 76).

Other studies track dispersal in wind by capturing spores. Peay et al. (30, 77) documented the dispersal of ectomycorrhizal assemblages at least 10 kilometers away from their source by placing uninfected "trap" seedlings at different distances from a source population. Data reveal that species richness and trap seedling colonization drop significantly beyond 1 kilometer. Additionally, viable ascospores of the wheat pathogen *F. graminearum* have been captured 50 meters to 1 kilometer above the Earth's surface in all seasons in Virginia—even during winter, when its plant host is absent. The capture of spores during winter suggests that the source of spores is kilometers away, because there was no wheat in the vicinity of the experimental setup when the *F. graminearum* spores were collected (68, 70, 71).

The most complete picture of wind LDD emerges from research on *A. sydowii*, the causal agent of aspergillosis of the Caribbean sea fan, *Gorgonia ventalina*. An outbreak of the disease occurred during the 1980s and coincided with the highest recorded deposition of African dust in the Caribbean Sea (42). Air samples taken from African dust plumes revealed the presence of *A. sydowii* conidia, and by inoculating *G. ventalina* in laboratory assays, these same dust-borne conidia were shown to cause the same symptoms of aspergillosis as occurring in the Caribbean (42, 67). Furthermore, only samples derived from African air masses moving over the Caribbean contained viable *A. sydowii* material, while samples from air masses of different origins did not (78).


Defining and Comparing the Dispersal Kernels of Fungi
Two idealized dispersal kernels
Density of Spores Reaching a Particular Distance
Spore Source
(e.g. a single mushroom)
Distance beyond which only 1% of spores travel and beyond which LDD is defined
Furthest dispersal distance
Dispersal Distance
Mycosphaerella fijiensis
No. of Resistant Lesions / m2 of Leaf
Fusarium graminearum
Recovery Rate of Released Clone
Lobaria pulmonaria
Number of Positive Samples
Distance (m)

Atmospheric LDD may involve more than wind and may be facilitated by a combination of meteorological phenomena, including cloud, storms, and precipitation. In fact, spores may serve as rain- and cloud-forming nuclei, although the limited evidence for this phenomenon is debated, and it is still unclear how water can condense on the potentially hydrophobic outer surface of spores (see section below on morphological, biophysical, and physiological properties influencing LDD).

Fire may also play a role in initiating LDD because it can rapidly heat air and cause high-velocity updrafts (79). For example, back trajectory modeling of air masses suggests that viable spores captured from smoke over the Gulf Coast of Texas originated 1,500 km away in forest fires in the Yucatán Peninsula, Mexico (80). Sugarcane agriculture provides an excellent model for exploring how fire promotes LDD, because fields are frequently burned prior to harvest and are often plagued by one of the most widely referenced putative long-distance dispersers, *P. melanocephala*—among the first fungal species described as having undergone trans-Atlantic dispersal (79, 81). Fire-borne updrafts, perhaps often caused by humans, may facilitate the spread of *P. melanocephala*, challenging hypotheses of the unassisted dispersal of spores across oceans (cf. 73, 74).

Plants

Plants are another agent of fungal dispersal, and their ability to vector fungi is unsurprising given the close ecological association between the two kingdoms. Fungi inhabit both living and dead plant tissues, and there are many opportunities for fungi to codisperse, e.g., with seeds, senesced leaves, or branches.

Driftwood is an often overlooked substrate in which fungi disperse. Saprotrophic fungi are often found in decaying logs floating in bodies of water, and if hyphae or spores are able to withstand saline conditions, driftwood may be able to transport species across oceans. For example, Rämä et al. (82) sampled logs from across the North Sea and successfully cultured 147 fungal operational taxonomic units of Ascomycota, Basidiomycota, Mucormycotina, and Chytridiomycota, 50% of which were identified as terrestrial (nonmarine). Driftwood kept afloat by ice flows during the late Weichselian or early Holocene is suggested as a mediator of LDD for several trans-Arctic plant species and likely also their fungal symbionts (83). Data already support the long-distance movement of driftwood; e.g., Hellman et al. (84) show that logs collected in Greenland and Svalbard originated from western and central Siberia and North America. However, the

Figure 3 To integrate the disparate approaches used to describe and measure fungal LDD, we propose a synthetic three-part definition building on the general framework presented by Nathan (1, 28). A description of fungal LDD should include (i) identification of a source population and measure of source inoculum concentration (e.g., the number of spores in a single rust pustule), (ii) a measured and/or modeled dispersal kernel, and (iii) a measure of the distance, based on the dispersal kernel, past which only 1% of spores travel. Adopting a standard approach would mitigate the confusion caused by differing definitions and measurements of LDD and facilitate comparisons among the dispersal kernels of different species. In the illustration, the blue and red dispersal kernels demonstrate idealized kernels for two hypothetical species. LDD is defined per species at distances A and B, respectively—the distance beyond which only 1% of spores travel. We next used our approach with real dispersal data of *M. fijiensis* (measured as the number of resistant lesions per square meter of banana leaf measured from a source to 1,000 m) (52), *Fusarium graminearum* (measured as the recovery rate of ascospores of a unique clone released from a source to 1,000 m) (58), and *Lobaria pulmonaria* (measured as the proportion of DNA from snow samples identical to an isolated source of soredia up to a distance of 40 m [193]) to estimate dispersal kernels and identify LDD for each species. We smoothed the published data to estimate an approximate dispersal kernel, and the distance beyond which 1% of spores traveled was found by integrating the area under each kernel from 0 m to the distance at which 99% of spores had been captured. Although both *M. fijiensis* and *F. graminearum* are capable of dispersing to approximately 1,000 m, the proportion of spores that fit our definition of LDD varies considerably, because LDD is defined past 714 m for *F. graminearum* and past 250 m for *M. fijiensis*. A holistic comparison of the two dispersal kernels suggests that different dynamics will shape the effective reach of each species. The dispersal kernel of *L. pulmonaria* illustrates how truncated experimental setups can impact measures of LDD. At the furthest collection point (40 m), a large proportion of samples tested positive, and the best dispersal kernel that can be modeled from the data (193) provides what is likely an underestimate of LDD, at approximately 39 m (15% of the positive samples collected at 0 m were detected). Ideally, the tail end of a modeled dispersal kernel should very closely approach a horizontal line at $y = 0$.

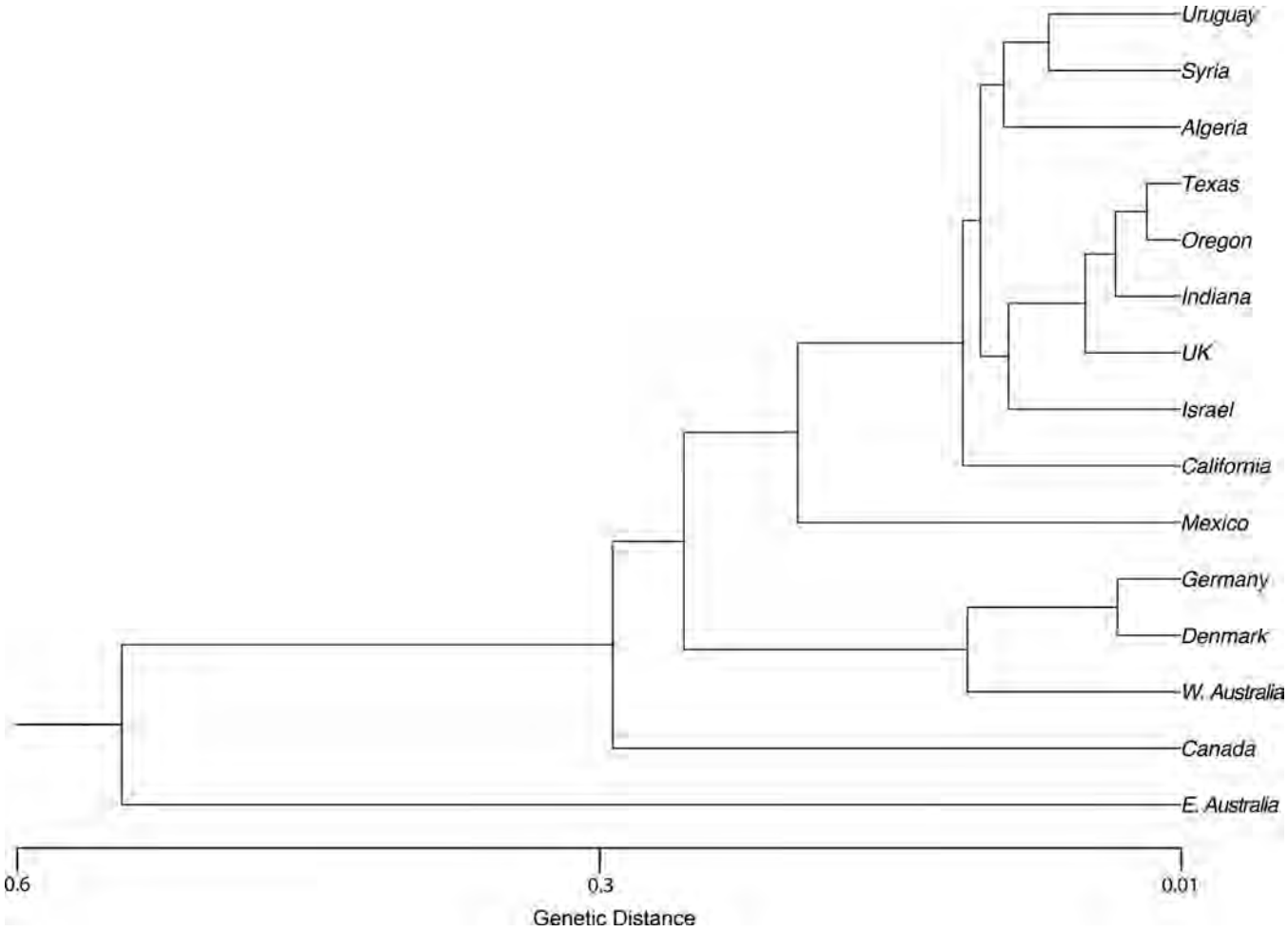


Figure 4 A phylogram of genetic distances among 15 geographic populations of *Mycosphaerella graminicola*. The fact that geographically distant populations of *M. graminicola* are grouped together, e.g., Uruguayan populations are grouped with Algerian and Syrian populations, likely suggests movement mediated by humans. *M. graminicola* infects one of the most traded agricultural products (wheat), and its ascospores cannot survive prolonged exposure to, e.g., dry air (183). Data adapted from Zhan et al. (64); similar clustering of geographically distant populations is found from data on *Phaeosphaeria nodorum* (129), *Rhynchosporium secalis* (131), and *M. fijiensis* (60).

majority of wood was logged, again suggesting that humans play a key role in many different kinds of LDD. The problem of driftwood-associated fungi remains a promising area for future research, and open questions concern patterns of driftwood movement and their possible relationship to fungal introductions and whether some logging practices increase the likelihood of LDD.

Living plant material transported by ocean currents is another putative mediator of fungal LDD. Symbiotic fungi are associated with plant roots as mycorrhizae and with leaves, stems, and seeds as endophytes. Thus, ocean-dispersed plant material, including floating seeds, asexual propagules, or entire root balls, may explain the geographic range of some fungi that are found on two sides of, e.g., an ocean. Little to no direct evidence for this phenomenon has been collected to date, and phylogenetic analyses testing whether plants and fungi disperse together are, surprisingly, lacking (85). Anecdotal evidence of arbuscular mycorrhizae occurring most frequently and with greater biomass on Hawaiian endemic beach grass species has been used to suggest fungus-plant long distance codispersal (86, 87). However, Koske and Gemma (87) provide alternative hypotheses such as concurrent, independent dispersal and sea bird-mediated dispersal of arbuscular mycorrhizae. A codispersal hypothesis is also suggested as an explanation

for evidence of recent gene flow between island and mainland ectomycorrhizae, though vectors such as wind cannot be ruled out (85, 88).

Oceans, Rivers, and Lakes

Large bodies of water can act as vectors of fungal dispersal. Oceans and lakes provide large areas across which some fungi can freely travel by, e.g., moving with microcurrents and upwelling, while rivers and streams provide continuous movement in the direction of their flow. The number of fungi specifically adapted to an aquatic or amphibious lifestyle is estimated at more than 10,000 species, although only approximately 500 have been formally described (89). Aquatic species are informally divided into two major groups: Ingoldian fungi, found on decaying leaves in streams and lakes, and aquatic ascomycetes (traditionally referred to as hyphomycetes), found on submerged wood (90). The uncommon shapes of many aquatic fungal spores may facilitate dispersal, as well as adherence to various substrates, in aquatic environments. Conidia are typically sigmoid or tetraradiate, while ascospores are generally fusiform with bipolar mucilaginous pads (see Fig. 5N) (91).

Whether spores are passively or actively released remains unclear. Most marine ascomycetes appear to release their spores passively, while many tropical freshwater ascomycetes actively eject their spores away from the fungal thallus (92). A possible explanation for this pattern may involve wind dispersal of spores during seasonal drying of streams and rivers. Alternatively, storms can cause flooding and the accumulation of substrate on, e.g., riverbanks and subsequently expose ascocarps to airflows once waters subside (92). In both cases spores are hypothesized to be sometimes dispersed by air, although the interplay between aquatic and terrestrial habits of these kinds of fungi requires further study.

At least a few Ingoldian fungi appear to have cosmopolitan ranges, suggesting they may be capable of LDD (90). For example, recent phylogenetic methods have elucidated that there is no geographic structure to populations of the widely distributed marine fungus *Lignicola laevis*, hinting that the species may be a long-distance disperser. However, only two loci were used in the study, and including several more genetic regions may reveal more restricted population assemblages (75, 76, 89). Pang et al. (89) list several other species which seem to have similar cosmopolitan distributions—*Aniptodera cheasapeakensis*, *Ceriosporopsis halima*, *Corollospora maritima*, *Savoryella lignicola*, *Torpedospora radiata*, and *Zalerion maritima*—suggesting that aquatic fungal LDD may be an as yet undescribed phenomenon.

Aquatic environments may be an ideal place for LDD to occur considering that fungal dispersal is often limited by spore desiccation, UV damage, and harsh temperatures. Water temperature fluctuates more slowly than that of air, and water attenuates light penetration at relatively shallow depths (especially in highly trophic waters). Moreover, water provides a greater degree of buoyancy than air, increasing the time before spore sedimentation. Aquatic dispersal is perhaps the least commonly considered mechanism of LDD, and future studies might address the coupling of, e.g., spore hydrodynamics with river flow velocity, as well as the population structure of putatively cosmopolitan aquatic fungi.

Animals

Animals are also vectors of fungal dispersal. Many animals migrate across continents on an annual basis and may transport fungi either internally or externally as spores, hyphae, sclerotia, or symbionts (93–98). Fungal propagules sheltered deep within fur or feathers are potentially protected from some harsh environments as they move over large distances.

Flying animals clearly serve as fungal vectors, and there is a great deal of evidence for birds and insects as mediators of fungal dispersal, especially of pathogens. Examples in arthropods include the spread of *Entomophaga maimaiga*, an introduced pathogen of gypsy moths used for biocontrol in North America (99); *Aspergillus flavus*, which infects desert locusts in India (100); *Sphaeropsis sapinea*, a pathogen of conifers worldwide that is spread by the pine engraver beetle (101); *Ophiostoma* spp. and *Knoxdaviesia proteae*, commensal species of mites secondarily vectored by beetles in South Africa (102, 103); and many others. However, dispersal by insect vectors tends to be restricted within a localized range, e.g., a few hundred kilometers, while dispersal across, e.g., continents or oceans, is more commonly caused by the human-mediated movement of insects and fungi together (103, 104).

Migrating birds are another common agent of animal-mediated dispersal. Examples include *Gibberella fujikuroi* (*Fusarium moniliforme*), a pathogen of rice vectored by hummingbirds (105); *Encephalitozoon* and *Enterocytozoon* spp., microsporidian human pathogens collected from several bird species (106, 107); and 2,337 filamentous fungi isolated from 216 migrating Mediterranean birds, of which *Cladosporium cladosporioides*, *Alternaria alternata*, and *Aspergillus niger* were the most abundant (108).

The recent spread of white-nose syndrome in North America, caused by *Pseudogymnoascus destructans*


A
300µm
B
15µm
C
10µm
D
10µm
E
10µm
F
1µm
G
5µm
H
10µm
I
5µm
J
10µm
K
5µm
L
3µm
M
3µm
N
10µm

(*Geomyces destructans*), is another example of flying animal-mediated fungal dispersal. The mycosis appears to be spread among congregating bats and by their subsequent movement to other caves (109). The recent emergence of the disease in North America has resulted in the death of millions of bats, but it is unclear if the epidemic has resulted from the introduction of a European species or from the recent emergence of a newly virulent North American strain (110). In either case, the disease is spreading on a continental scale, and in addition to bats, humans may play a role in its spread (109).

Finally, the spread of chytridiomycosis of amphibians, caused by *Batrachochytrium dendrobatidis*, is perhaps the most commonly cited example of putative animal-mediated LDD. In recent years chytridiomycosis has spread rapidly, perhaps facilitated by a changing climate, as shown in Central America (111). The disease is heterogeneously distributed across all continents except Antarctica, but the reasons for its disjointed distribution are unknown (112, 113). It is not entirely clear how the fungus moves over large spatial scales, but its spread may be caused by a combination of localized amphibian movement coupled with, again, human mediation via the international trade of *Xenopus laevis* (the African clawed frog) and other amphibians (114–116).

Humans

Ancient fungal dispersal mediated by human migrations is suggested by data on population structures (117–119). The range expansion of the fungal pathogen *Coccidioides immitis* into South America parallels human migration routes during the Pleistocene (120–122). Similarly, the diversification of *Saccharomyces cerevisiae* strains mirrors their use and movement with human populations (123).

Contemporary dispersal mediated by human vectors merits special consideration as the inadvertent transportation of biological materials continues at unprecedented spatial and temporal scales. Plant disease epidemics caused by introduced fungal pathogens are among the clearest examples of the impact of human-mediated LDD, e.g., *Cryphonectria parasitica* (chestnut blight), *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* (Dutch elm disease), and *Cronartium ribicola* (white pine blister rust) (124–127). But human-mediated LDD is not restricted to pathogens. For example, Vellinga et al. (128) show that many genera of ectomycorrhizae have also been introduced to novel ranges and spread globally by the human movement of plants and soil.

The global transport of agricultural products, as well as exotic plants, animals, and soil, all serve either indirectly or directly as platforms by which fungi can disperse over large distances at an accelerating rate. Modern transportation enables fungi—including fungal tissue that cannot independently disperse—to traverse continents in less than 24 hours. For instance, fruits and vegetables grown in North America typically spend a maximum of 5 days in intracontinental transit following harvest, and the transport time of produce grown in the Southern Hemisphere for U.S. consumption can take as little as a few days, depending on the mode of transportation (46).

Although many examples of human-mediated fungal dispersal are well documented, circumstantial evidence points to an even greater array of human-mediated dispersal events that are less well understood. Examples include *M. fijiensis* (black sigatoka) and *Mycosphaerella graminicola* (septoria leaf blotch), *Puccinia striiformis* f. sp. *tritici* (wheat yellow rust), *P. melanocephala* (sugarcane rust), *H. vastatrix* (coffee rust), and *Rhynchosporium secalis* (barley scald) (59, 63, 64, 73, 74, 129–131). Many of these species are intimately associated with agriculture, are planted over vast areas, and are regularly moved (either superficially on or within plant tissue) on a global scale. These same species are also frequently cited as prime examples of fungi capable of LDD (25). However, few to no data on fungal characteristics enabling or inhibiting LDD are available;

Figure 5 Images of various fungal spores. (**A**) Basidiospores of *Agaricus bisporus* (brown powder) next to seeds of *Wolffia borealis* (semicircles) and sugar crystals (white cubes). (**B**) Urediniospores of *Puccinia menthae* (Fig. 1 of reference 194). Conidia of (**C**) *Alternaria solani* and (**D**)*A. alternata*. *A. alternata* is a putative long-distance disperser, while *A. solani* (10× in size) is not (courtesy of Steve Jordan). (**E**) Glomerospore of *Glomus irregulare* (Fig. 5i of reference 195). (**F**) Conidium of *C. herbarum* (Fig. 5c of reference 196). (**G**) Teliospore of *Tilletia controversa* (Fig. 9 of reference 197). (**H**) Urediniospore of *H. vastatrix* (Fig. 1e of reference 198). (**I**) Urediniospore size, shape, and ornamentation of *P. melanocephala* (Fig. 1d of reference 199). (**J**) Zoospores of chytrid *Rhizophydium elyensis* (200). (**K**) Ascospores of *Ascobolus denudatus* (200). (**L**) Sporangiospores of *Rhizopus microsporus* var. *chinensis* (200). (**M**) Basidiospores of *Boletellus taiwanensis* still on soredia (200). (**N**) Conidia of the aquatic ascomycete *Nawawi dendroides* (Fig. 66 of reference 92).

to travel, e.g., across oceans, spores must presumably surmount considerable biophysical constraints. Many of these pathogens are also globally distributed—as are their crop hosts—and an alternative hypothesis explaining what appears as LDD would involve a global network of commerce that provides multiple opportunities for infectious material to be transported between locations.

Consider the global populations of *M. graminicola* studied by Zhan et al. (64), of which, e.g., populations in Syria and Uruguay are genetically less distant from each other than geographically close populations sampled from both eastern and western Australia (Fig. 4). The fact that geographically distant populations of *M. graminicola* are grouped together, e.g., Uruguayan populations are grouped with Algerian and Syrian populations, suggests movement by humans. *M. graminicola* infects a highly traded agricultural product, wheat, and its ascospores cannot survive prolonged exposure to, e.g., dry air (132). However, with enough time, if gene flow were to completely halt, geographic populations could diverge and no longer appear as nested populations, although relationships between genetic and geographic distances would remain difficult to interpret. Highlighting the connection between human-mediated LDD and its effects on population structure may prove itself as a key variable to consider when trying to determine vectors and mechanisms of fungal LDD.

MORPHOLOGICAL, BIOPHYSICAL, AND PHYSIOLOGICAL PROPERTIES INFLUENCING LDD

Spore Size and Shape

The most obvious and perhaps most important agent of dispersal is the spore, whose size and shape may critically affect movement over large distances (Fig. 5) (133). A spore's ability to reach airflows, remain aloft, and then land in a suitable location is influenced by aerodynamic forces operating at a microscopic scale, and such forces may be harnessed by manipulating spore morphology (133, 134). Although Jenkins et al. (135) report no correlation between propagule size and dispersal distance in general cases, aspects of spore morphology are clearly optimized for movement. For example, Fritz et al. (136) have shown that among some Ascomycetes, spore dimensions precisely fit apical ring size to maximize launch distance with minimal energy. Others show that spore size can also be correlated to environmental parameters in ways that might maximize the probability of LDD. For example, Kauserud et al. (137, 138) show a relationship between spore size and the calendar date of sporulation and reason that spore morphology enables some fungi to take full advantage of seasonal wind velocities. However, when we compiled data on spore sizes and dispersal distances claimed as fungal LDD (Table 1) we found no relationship between spore morphology and dispersal distance (Fig. 6), but we hypothesize that the lack of any apparent correlation reflects the different measures and definitions of LDD used in the literature and not necessarily the lack of a biological relationship.

There is likely a compromise between small spore size, which can enable dispersal over longer distances, and large spore size, which can facilitate settling onto a favorable substrate (Fig. 5C, D). In principle, smaller spores should remain aloft for greater time intervals, but their reduced mass makes landing more difficult and increases their susceptibility to adverse environmental pressures, including UV exposure and desiccation (139). Greater and improved data on a range of spore parameters—emphasizing spore size, shape, longevity, and density—are required to further explore the tradeoffs involved in successful LDD. Often, the aerodynamic diameter (defined as the diameter of a spherical particle with equal density and terminal velocity to the particle of interest) of a spore is the sole parameter considered in estimates of spore dispersal (140). The focus on aerodynamic diameter may be problematic because many spores are not spheres, and also because density measurements specific to species of interest are not available but are necessary for accurate extrapolations of dispersal in heterogeneous airflows (140–142).

Successful fungal dispersal appears also to rely on a critical interplay between drag reduction (to maximize launch height) and drag maintenance (to maximize flight time). Roper et al. (143) have shown that explosively launched spores of many Ascomycetes have drag-minimizing shapes. Drag minimization enables spores to breach the boundary layer of still air surrounding sporocarps to reach more turbulent air layers. However, once aerosolized, successful LDD may require spores to remain aloft for extended periods (133). Wong et al. (144) have shown that remaining aloft is more a function of spore volume than of shape and have observed that the drag constants of spores are surprisingly proportional to their surface area (discounting shape and type of particle). Therefore, spore size appears to have, overall, a greater effect on settling velocity than do shape and density, suggesting that the latter characteristics may be less important in determining sedimentation rates (140, 142). An exciting direction for future

Table 1 Spore parameters for putative long-distance dispersers

Putative LDD fungal species	Spore type	Spore dimensions[a]	Shape	Habit	Pigment	Clump	Reference
B. graminis f. sp. *tritici*	Ascospore	20–30 μm (l) × 10–13 μm (w)	Ellipsoid	Plant pathogen	Hyaline	Yes	201
Gibberella zeae/Fusarium graminearum	Ascospore	13–28 μm (l) × ~4 μm (w)	Long ellipsoid	Plant pathogen	Light brown to hyaline		202
M. fijiensis	Ascospore	11.5–16.5 μm (l) × 2.5–5 μm (w)	Fusiform	Plant pathogen	Hyaline		41
M. graminicola/Septoria tritici	Ascospore	8–10 μm (l) × 2–2.5 μm (w)	Fusiform	Plant pathogen	Hyaline to light brown		65
Mycosphaerella musicola	Ascospore	14.9 μm (l) × 4.6 μm (w)	Fusiform	Plant pathogen	Dark brown		203
Phaeosphaeria nodorum	Ascospore	20–31 μm (l) × 4–5 μm (w)	Fusiform	Plant pathogen	Yellow-brown		129
Sclerotinia sclerotiorum	Ascospore	12 μm (l) × 6 μm (w)	Ellipsoid	Plant pathogen	Hyaline	Yes	204
Venturia inaequalis	Ascospore	11–15 μm (l) × 5–7 μm (w)	Ellipsoid	Plant pathogen	Brown		205
G. applanatum-australe	Basidiospore	Applanatum: 6.5–8.5 μm (l) × 4.5–6 μm (w); australe: 8–13 μm (l) × 5.5–9 μm (w)	Ellipsoid	Saprotroph	Brown		88
Laccaria amethystina	Basidiospore	6.16–8.47 μm (diam)	Globose, ornamented	Mycorrhiza	White		206
A. alternata	Conidia	20–63 μm (l) × 9–8 μm (w); often produced in chain of more than 5 conidia	Obclavate to obpyriform	Plant pathogen	Beige to brown	Yes	19
A. sydowii	Conidia	2.5–4.0 μm (diam)	Globose	Animal biotroph	Hyaline		68
C. herbarum	Conidia	1–12 μm (l) × 1–10 μm (w) with 50–100 nm × 50–400-nm bundle-like structures	Ellipsoid	Plant pathogen	Melanized	Yes	19
R. secalis	Conidia	12–20 μm (l) × 2–4 μm (w)	Fusiform	Plant pathogen	Hyaline		131
Peronospora hyoscyami f.sp. *tabacina*†	Oospore	17–28 μm (l) × 13–17 μm (w)	Globose	Plant pathogen	Hyaline		207
Sporisorium scitamineum	Teliospore	5 μm (diam)	Ovoid	Plant pathogen	Brown	Yes	208
Ustilago nuda	Teliospore	6.5 μm (l) × 5.8 μm (w)	Subglobose	Plant pathogen	Golden brown		197
H. vastatrix	Urediniospore	29.7–34.5 μm (l) × 18.9–37.3 μm (w)	Ellipsoid	Plant pathogen	Yellow	Yes	198
Phakospora pachyrhizi	Urediniospore	18–34 μm (l) × 15–24 μm (w)	Globose	Plant pathogen	Pale yellow to hyaline	Yes	209
P. graminis f.sp. *tritici*	Urediniospore	28.3 μm (l) × 17.5 μm (w)	Globose	Plant pathogen	Brown	Yes	210
P. melanocephala	Urediniospore	28–33 μm (l) × 18–23 μm (w)	Obovoid	Plant pathogen	Cinnamon brown		162
P. striiformis f.sp. *tritici*	Urediniospore	14–36 μm (l) × 13–23 μm (w)	Ellipsoid	Plant pathogen	Yellow to brown	Yes	211
B. dendrobatidis	Zoospore	3–5 μm (diam); posterior flagellum (19–20 μm long)	Ovoid	Animal pathogen	Hyaline		212

[a]Dimensions are given for major (l) and minor (w) axes or, when spherical, for diameter (diam). †, oomycete.

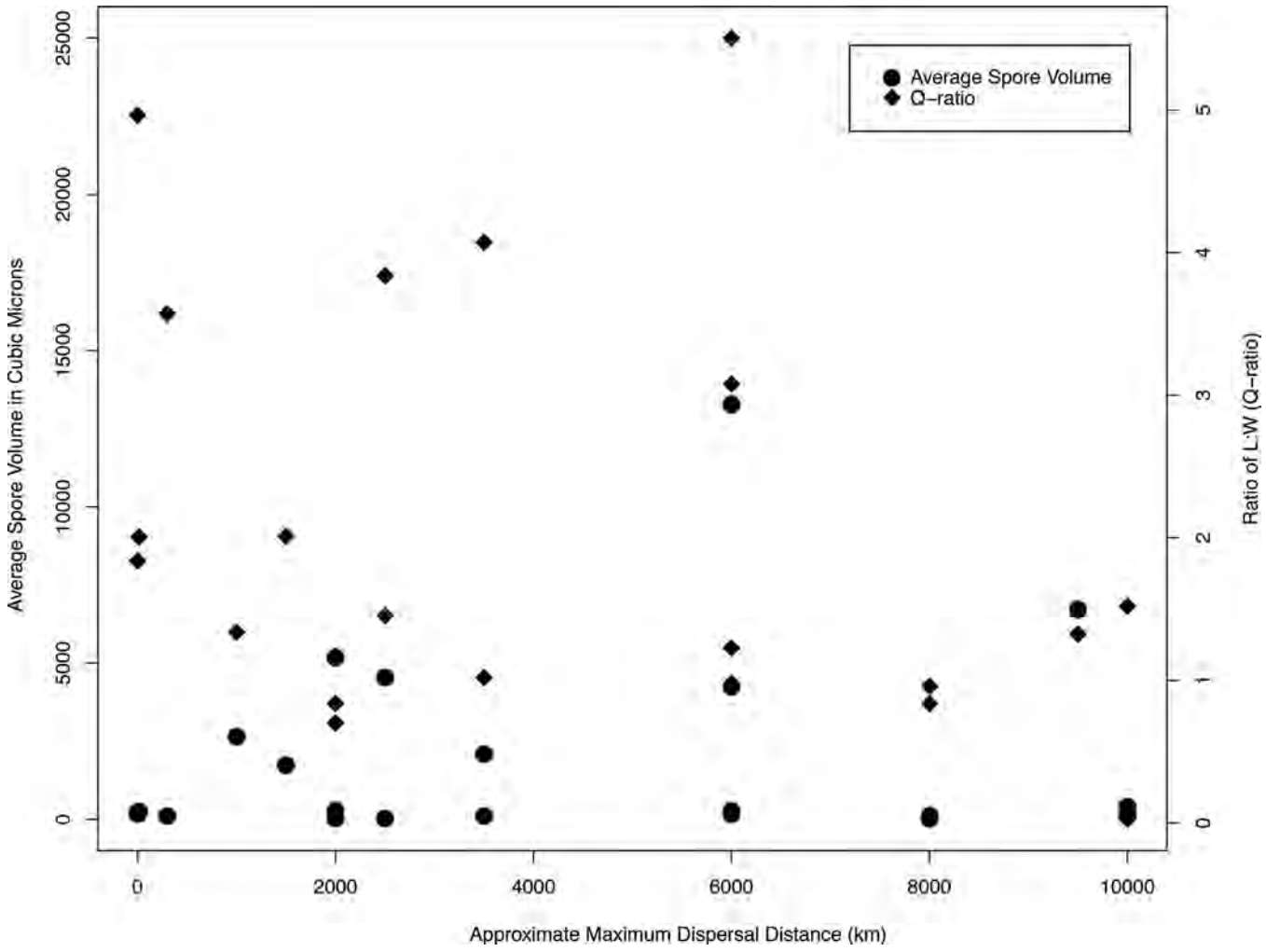


Figure 6 Comparing spore sizes to reported maximum dispersal distances. Spore volume in square micrometers is measured on the left-hand vertical axis, and spore Q-ratio (the ratio of spore length to width) is measured on the right. Data points were calculated from the parameters listed in Table 1. There is a poor correlation between approximate maximum dispersal distance and both average spore volumes ($R^2 = 0.0167$, $P = 0.5568$) and Q-ratios ($R^2 = 0.1113$, $P = 0.1198$). The lack of any correlation likely reflects inconsistent definitions and measurements of LDD, rather than any biological reality.

research involves more thorough testing of whether or how fungi have adapted to take advantage of aerodynamic principles, especially among putative long-distance dispersers.

A Spore's External Surface

Additional aspects of morphology that may influence spore dispersal include ornamentation and hydrophobicity, although these features appear to be more rarely studied than shape and size, despite limited data suggesting their key role in dispersal. For example, Halbwachs et al. (145) report that asymbiotic agaric species tend to be more ornamented than ectomycorrhizal agarics, while the latter tend to have smoother, more pigmented spore walls; differences may reflect distinct dispersal dynamics; e.g., ectomycorrhizae may require more pigmentation for UV protection while dispersing greater distances to find a plant host (but see reference 146 for criticisms related to methodology). The many unanswered questions surrounding ornamentation and its potential impact on dispersal include, How does a spore's outer morphology affect spore-to-spore aggregation, surface impaction, and dry or wet deposition (147) (Fig. 2)?

Slightly more is known about spore surface hydrophobicity. Aimanianda et al. (148) have shown that hydrophobic surface proteins on fungal spore walls allow many species to remain dormant inside animal lungs without causing an immune response. While spores rarely escape from lungs, spore hydrophobicity may protect spores in other animal cavities, e.g., the gut, and hydrophobins may enable survival over the relatively large distances covered by many animals. If spores remain undetected and viable in animal digestion tracts until excretion, spore hydrophobicity may well play a role in long-range movement of fungi within animals (149, 150).

Spore surface hydrophobicity also raises questions about whether spores can play a role in meteorological phenomena, either as cloud-condensing nuclei or ice nuclei (143, 151, 152). Spores can theoretically disperse within cloud formations, e.g., at the core of an ice particle, but how water would condense on hydrophobic spore walls remains an open question (153–155). Some kinds of plant pollen do act as cloud-condensing nuclei in high-humidity environments, despite a waxy outer layer. Pope (156) hypothesizes that the small pores found on pollen surfaces (approximately 1 μm in diameter) cause a localized reduction in vapor pressure and, as a result, capillary condensation. Similar morphologies are seen on ornamented fungal spores and, famously, on the adaxial surface of many basidiospores (143, 157–159). These structures may affect nucleation, although to date no study has tested whether spore wall ornamentation drives water condensation.

Do Spores "Clump?"

Dispersal may also be influenced by the ability of spores to aggregate, or form clumps. Clumping is reported for a variety of species, including *P. graminis*, *P. striiformis*, *A. alternata* (Fig. 5D), *Cladosporium herbarum* (Fig. 5F), *Blumeria graminis*, *Phakopsora pachyrhizi* (Fig. 5I), and *H. vastatrix* (Fig. 5H) (160–163). Clumping may improve individual fitness by stimulating germination (164) and may facilitate impaction on substrates by providing greater inertial mass (150, 165). Moreover, the outermost spores in airborne clumps may shield the innermost spores from harmful environmental conditions, e.g., solar radiation (163). However, whether clumping provides a net benefit to spores remains unknown. While the lower mass of a single spore may facilitate launch into turbulent air layers, the greater mass of clumped spores may shape horizontal displacement and deposition. Moreover, data on clumping are limited to a handful of fungal species (164, 165), and more research is needed to understand when, how, and how commonly spore clumps form.

The Physiological Hardiness of Spores

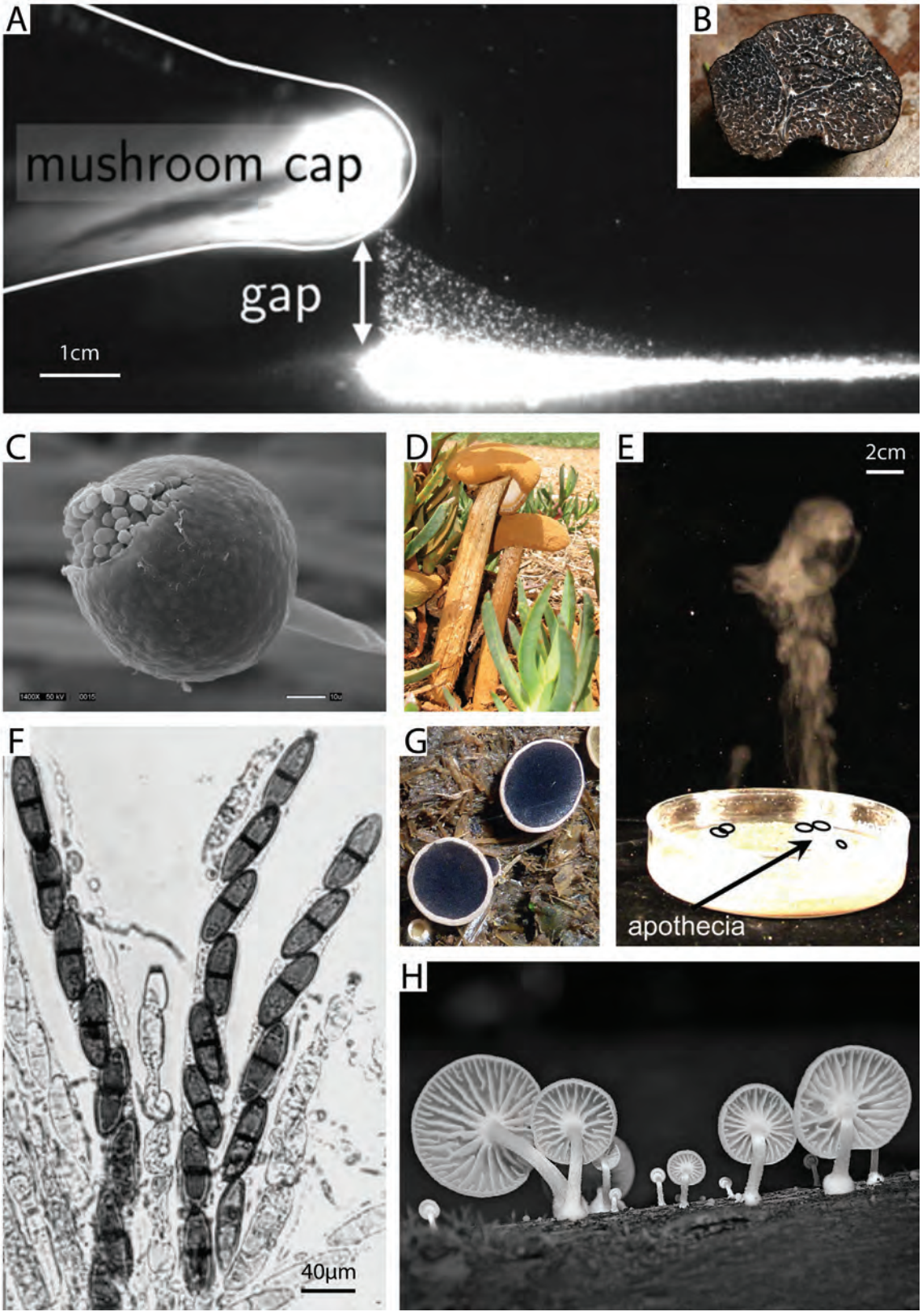
Fungal LDD may be constrained by the physiological tolerances of spores to solar radiation (especially UV), air moisture (relative humidity), and temperature. The resilience of a spore to any of these variables will vary according to species or taxonomic group. For example, urediniospores of *P. striiformis* var. *tritici* die quickly when exposed to high solar radiation, while ascospores of *Gibberrella zeae* are more susceptible to low relative humidities, and urediniospores of *P. pachyrhizi* cannot tolerate cold temperatures (166–168).

Because of the diversity of physiologies involved, the ability of species to withstand stresses must be tested individually. For example, if a species is hypothesized to have traveled from West Africa to Brazil by wind (cf. 73), verifying whether the spores of that particular species can withstand the UV, relative humidities, and temperatures likely encountered over the predicted path of flight is a critical and simple check on the plausibility of LDD. These kinds of data would complement evidence of inferred LDD, e.g., population genetics data, and enable a more comprehensive understanding of the likelihood of dispersal.

Sporocarp Properties Influencing LDD

Whether sporocarps influence LDD depends on the context in which dispersal occurs. Fungal species whose dispersal is animal-mediated, whether externally or internally, have evolved sporocarps with specific mechanisms to attract vectors. For example, *Tuber* spp. synthesize volatiles to encourage fungivory by mammals, while *Phallus* spp. produce foul aromas to attract insects (169–173).

Intriguingly, recent evidence suggests that sporocarps also play an active role in mediating wind dispersal. Within Ascomycetes, despite the diversity of spore and ascus apical ring shapes involved, the launch velocity of 90% of ascospores is within 2% of optimal energy conservation, so the morphology of the ascus facilitates ascospore penetration beyond the boundary layer of still air surrounding an ascocarp (Fig. 7F) (133, 136). Apothecia can also synchronize the release of spores, enabling groups of spores to move through still air to heights that could not be reached by the forcible discharge of a single spore (134). Basidiomycete mushrooms also appear to manipulate dispersal by using water evaporation from the pileus to generate convective airflows and move spores by at least several centimeters vertically (Fig. 7A) (174).


A
mushroom cap
gap
1cm
B
C
D
E
2cm
apothecia
F
40µm
G
H

Less clear is whether sporocarps can control the timing of spore release to take advantage of local weather that might enhance the probability of LDD. Using a Lagrangian stochastic model, Savage et al. (175) show that fungal spores released during the hottest times of day are most likely to undergo LDD, presumably because updrafts formed by heated low-altitude air masses can lift spores into turbulent flows at higher altitudes. Extreme weather events, including thunderstorms and tornados, can also generate intense vertical updrafts and lift air into the upper troposphere. Anecdotal evidence suggests that fungi may release a greater concentration of spores just before thunderstorms (when updrafts are prevalent), and there are records of asthma outbreaks caused by fungal spores specifically prior to thunderstorms (40, 176–179). Efforts to track the timing and number of spores released during atmospheric updrafts, and in relation to other meteorological phenomena, remain an interesting direction for future research and may offer additional perspectives on the ability of fungi to manipulate LDD.

UNKNOWN AND CONFOUNDING VARIABLES: FREQUENCY OF LDD, VICARIANCE, AND CHANGING PATTERNS OF HUMAN-MEDIATED DISPERSAL

The frequency of LDD for any particular species often remains unknown. Frequent LDD requires movement to be unhindered by (i) physical barriers, (ii) lack of vectors, or (iii) unsuitable habitat. If LDD is frequent enough and occurs on a global scale, it results in what appears as a global population structure (32). Rare LDD might involve a single stochastic founding event and would be reflected in population structures where shared alleles become rare over time. Different rates of LDD are important because they result in very different dynamics when, e.g., a novel adaptive mutant (e.g., a genotype able to take advantage of a novel host) arises in one population.

When population structure is determined by geological events, rather than the movement of organisms themselves, the concept of vicariance is often invoked. Vicariance is typically defined as the fragmentation of a single population by changes in a landscape, causing limited to no gene flow between the resulting disjunct populations (180–183). Vicariance is relevant to a discussion of LDD because when data do not suggest vicariance, LDD often emerges as the default explanation for population structures. If populations appear related, despite a clear physical barrier (e.g., an ocean separating populations of the same species on two continents), LDD is often hypothesized to be the process causing the observed population structures (60, 184–187) (Fig. 4).

As discussed previously, when LDD is inferred from genetic data, the mechanism of LDD remains unknown. Natural vectors, especially wind, are typically invoked as an explanation, but the literature on vicariance and LDD may provide strong indirect evidence for the role humans play in mediating dispersal across extreme physical barriers, e.g., oceans and mountain ranges. This hypothesis is seldom discussed, but the inability of physical and/or genetic data to explain contemporary population structures for many species is highly suggestive of humans playing an expanding role in fungal LDD (20, 129, 131). As the entire field of invasion biology attests, there are different dynamics at play when humans become involved in mediating dispersal (188). Technology (e.g., commercial aircraft, ocean vessels, etc.) facilitates the movement of goods, and in recent times the spatial and temporal scales of potential fungal dispersal have clearly amplified. Understanding whether apparent gene flow is a function of changing human behaviors, and not part of an autonomous pattern more typical of the past thousands or millions of years, would usefully inform our understanding of, e.g., disease and changing patterns of biodiversity.

CONCLUSIONS: UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

Many unanswered questions remain, including, What shapes the end stages of successful LDD (defined as the

Figure 7 Images of spore dispersal structures among fungi. (**A**) Basidiospores of *Lentinula edodes* carried vertically by evaporative airflows from mushroom cap (Fig. 1e of reference 174). (**B**) Hypogeous spore body of *Tuber brumale* (Wikimedia Commons Creative Commons Attribution-Share Alike 3.0 Unported [WC]). (**C**) Sporangium of *Rhizopus oryzae* releasing sporangiospores (courtesy of Andrii Gryganskyi). (**D**)*Battarrea phalloides* mushroom (Doug Collins, WC). (**E**) Synchronous spore release from *Sclerotinia sclerotiorum* apothecia (Fig. 1b of reference 135). (**F**) Asci of *Amphisphaeria saccharicola* (200). (**G**) Apothecia of *Ascobolus scatigenus* (200). (**H**) Typical gilled agaric mushroom with gills to increase surface area of spore-producing tissue (WC).

growth and reproduction of an individual following its dispersal) (Fig. 1)? Dispersal can mitigate intraspecific competition and parent-offspring conflict, but dispersal to new habitats also involves demographic risks, e.g., the lack of mates, inbreeding depression, and other problems of small populations. Difficulties may be especially acute for individuals establishing at the tail end of a dispersal kernel. Other unanswered questions include, Which characteristics have evolved in some fungi to optimize the likelihood of LDD, and do some fungi take advantage of stochastic meteorological events, e.g., storms, or other local environments, to facilitate LDD?

Our definition of fungal LDD simplifies comparisons among species because it provides a relative measure of LDD for any species under consideration. LDD may involve tens of meters (e.g., a soil yeast) to kilometers (e.g., a smut fungus), and exact scales depend on the measured dispersal kernel for any given fungus. While our definition does not explicitly account for differences in the number of propagules found in, e.g., a mushroom, an infected plant, a field, etc., theoretically, a well-defined dispersal kernel will scale proportionately with the number of spores released. But whether the concentration of spores at a source affects the shape of a fungal dispersal kernel remains an open question. Greater numbers of spores may change the shape of a kernel if more spores increase the likelihood of, e.g., clumping, spore-to-spore wind entrainment, and wet deposition.

As global change affects the current ecological ranges of species, and biological materials continue to be moved on a global scale, defining LDD and understanding the mechanisms by which it occurs emerge as key research priorities. Fungal LDD has the potential to impact international food security and public health, as witnessed by emerging threats to soybeans in the United States and increasing cases of mucormycosis across the globe (132, 189–192). Human-mediated dispersal may drive current fungal LDD (cf. 28), though mycologists seem reluctant to mention the likely role of humans as dispersal agents. A mushrooming realization of the breadth of fungal biodiversity suggests that much is still to be learned about fungal dispersal.

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The Mycelium as a Network

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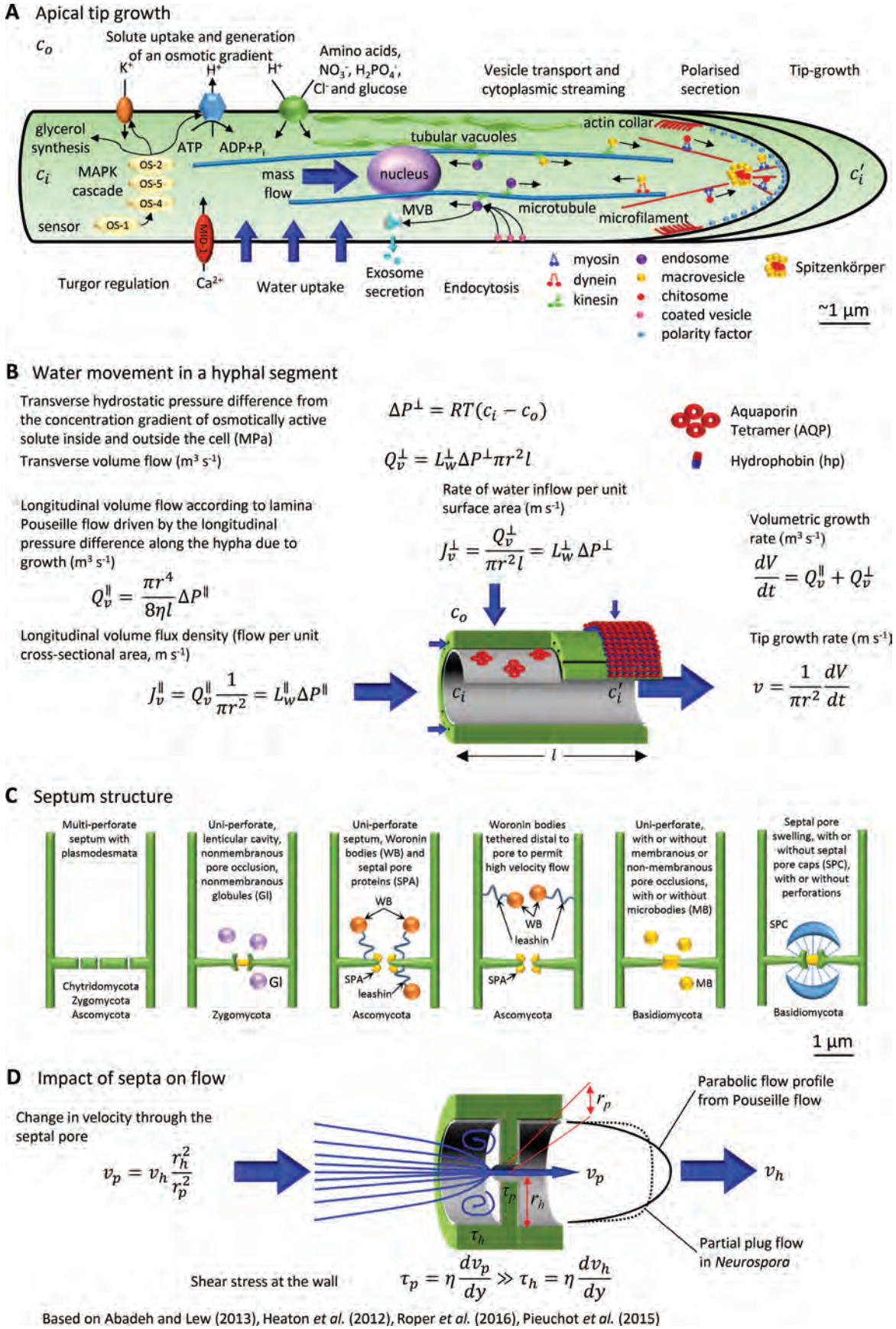
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INTRODUCTION

Growth as an interconnected mycelial network is characteristic of filamentous fungi and has been subject to scientific investigation since the seminal works of Buller at the start of the 20th century (1–3). We have increasingly detailed understanding of the fundamental cellular processes needed to form a network, such as hyphal tip growth (4), septation (5, 6), hyphal orientation (7), branching (8), and fusion (9–13) (Fig. 1A and C). In contrast, we know far less about the molecular events at the next physical scale that leads to hyphal aggregation and hyphal differentiation, and how these impact physiological processes such as long-distance resource distribution and biomass recycling. For example, while direct uptake and intrahyphal nutrient diffusion may be sufficient to sustain short-range local growth when resources are abundant (14), long-distance translocation is required to deliver nutrients at a sufficient rate to growing tips, particularly in fungi that form large networks on the forest floor that are too large to distribute nutrients through diffusion alone. We know little about the quantitative contribution of different potential transport pathways, such as cytoplasmic streaming, vesicle transport, growth-induced mass flow, or evaporative mass flow, to net fluxes and overall nutrient dynamics, and how they might vary between species and developmental stage (15–17). Nevertheless, the behavior of the growing mycelial network emerges from the interaction of many such processes and requires an integrated view to understand the overall impact on fungal behavior (18–20). Our understanding is further constrained by inferences drawn from a limited number of genetically tractable model filamentous species grown under laboratory conditions (abundant, evenly dispersed, low-molecular-weight resources, high relative humidity, constant light and temperature) compared with real-world conditions (patchy, recalcitrant, ephemeral resources, fluctuating temperature, light and relative humidity).

At the whole-colony level, there is a wealth of observational data describing fungal behavior (usually of non-model but ecologically relevant species) in qualitative terms, and some progress has been made in quantitative measurements of network architecture and dynamics, in parallel with development of predictive models for network function (21–33). The challenge is to construct a framework that can integrate information across scales into a coherent model to explain fungal behavior. We start this construction process by considering the biophysical properties of the mycelial network at each scale, with particular emphasis on the interplay between the network architecture and transport of resources through the network. Most of our knowledge on network structure and dynamics comes from non-resource-unit-restricted (i.e., fungi that can extend as mycelia from their food source) wood decay basidiomycetes that forage on the forest floor for resources distributed heterogeneously in space and time (34–36). We focus on these for discussion here for this reason, and because mycorrhizal networks have been comprehensively reviewed recently (37, 38). We also examine advances in image analysis techniques to extract the network architecture, empirically based modeling and simulation techniques to predict network behavior, and experimental approaches to map the actual functional flows within the network. We finally explore how network concepts may help to define a new set of fitness traits that can be experimentally determined (39).

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Based on Abadeh and Lew (2013), Heaton *et al.* (2012), Roper *et al.* (2016), Pieuchot *et al.* (2015)

THE BUILDING BLOCKS FOR NETWORK CONSTRUCTION

Growth at the Hyphal Tip

The mycelial network comprises individual hyphae ranging from about 2 to 20 μm in diameter that grow by tip expansion following highly localized polar secretion of wall materials (4) (Fig. 1A). Secretory vesicles are transported to the apical cell wall using a combination of microtubule and microfilaments, powered by molecular motors such as kinesin, dynein, and myosin (4, 40–42). Excess membrane is then recycled locally by endocytosis or sorted more extensively by motile early endosomes distal from the tip (40, 41, 43). Early endosomes may also play a role as a general transport system for other cellular components, such as ribosomes and mRNA (40, 44).

Motor-driven transport of vesicles and organelles concomitantly generates cytoplasmic streaming that serves to increase the effective transport rate for solutes and other cytoplasmic components (40). This is important because the synthesis of sufficient new wall material for maximal growth requires a minimum volume of cytoplasm distal to the tip (see "Modeling tip growth and branching" below) which has to be supplied by nutrient uptake and transport, often remote from the tip (42). Synthesis starts with accumulation of external ions, sugars, and amino acids present in the soil or following breakdown of organic substrates, using proton-coupled cotransporters or in response to the membrane potential via ion channels (Fig. 1A). The low intracellular water potential from solute accumulation leads to water uptake, which is required for the increase in hyphal volume during growth, and also leads to an increase in turgor pressure. Turgor is sensed by the conserved high-osmotic glycerol (HOG) mitogen-activated protein (MAP) kinase cascade and regulates plasma-membrane ion transport (Fig. 1A). In addition, osmolytes such as glycerol, arabitol, other polyols, proline, or trehalose may be synthesized internally to maintain turgor (45, 46). Stretch-activated plasma-membrane channels, such as Mid1, may respond directly to membrane stretch and also regulate solute uptake (Fig. 1A). For most fungi, some minimum turgor pressure is required for tip extension, but turgor pressure *per se* does not appear to be rate limiting for normal growth, because hyphae experiencing different turgor pressures can grow at the same rate. Indeed, some fungus-like oomycetes and wall-less fungal mutants can grow in the absence of turgor pressure, when the cytoskeleton alone may be sufficient to drive the tip of the hyphae forward (41, 47–50).

Transport at the Tip

Several parallel systems contribute to solute and organelle movement within the apical and subapical com-

Figure 1 Mycelial network formation: tip growth and fluid flows. (**A**) Hyphae grow by extension at the tip through polarized secretion of wall materials from macrovesicles and chitosomes at the apex, choreographed by the Spitzenkörper. Membrane is recycled distal to the tip into early endosomes by endocytosis. Endosomes may also form multivesicular bodies (MVBs) that may be involved in unconventional secretion of exosomes. Transport of secretory vesicles, endosomes, and other organelles along microtubule and microfilament networks also generates cytoplasmic streaming within the apical compartment. Although wall plasticity controls hyphal extension, the driving force involves maintenance of sufficient turgor pressure by water uptake through osmosis in response to accumulation of solutes. Turgor pressure may be sensed and regulated by a mitogen-activated protein kinase (MAPK) cascade or stretch-activated channels, such as mid1. If the site of water uptake required for the volume increase during growth is distal to the tip, growth-induced mass flows will also help to move organelles and solutes toward the tip. Based on references 4, 43, 49, and 64. (**B**) Water uptake into the hypha occurs to allow tip expansion and is driven by the transverse hydrostatic pressure in response to the difference in concentration of osmotically active solutes between the medium and the hypha. The transverse hydraulic conductivity depends on the permeability of the plasma membrane and aquaporins (AQPs) in parallel, and the wall and other surface layers, such as hydrophobins, in series. Longitudinal flow in the lumen of the hypha is lamina and follows Poiseuille flow. Based on references 32, 33, 49, and 79. (**C**) Variation in septal pore structure in different fungal taxa. Redrawn from reference 96 with permission. (**D**) Impact of the septa and septal pores on fluid flows. The change in cross-sectional area causes an increase in velocity by several orders of magnitude and also increases the wall shear stress within the pore. Flow may deviate from parabolic profile expected from the Hagen-Poiseuille equation because of the density of organelles in the cytoplasm. In addition, there may be eddy currents near the pore opening that trap nuclei, vacuoles, and other organelles. Based on references 32, 65, 68, and 69.

partments. In all systems, solutes will move by diffusion through the cytosol (51, 52) or the dynamic tubular vacuolar system (53–55), and this may be sufficient to allow bidirectional source-sink nutrient movements in slow-growing species (56), particularly as diffusion is constrained effectively to one dimension within the hyphae (52, 53), or "non-translocating" species that do not show protoplasmic streaming (57). For example, the time (t_D) for a species j to diffuse length l scales as l^2/D_j. Thus, ions and small molecules with a diffusion coefficient (D_j) of 1 to 2×10^{-9} m^2 s^{-1} would transit a typical apical compartment in *Phanerochaete velutina* (400 μm) in ~2 min, while proteins, with a diffusion coefficient of 2×10^{-11}, would take ~2 h. Transport over a small colony (10 mm) would take 20 h for small molecules and 7 weeks for proteins, and over a 25-cm microcosm transport would take 1.5 and 100 years, respectively. In practice, macromolecules are unlikely to have to move across the whole colony diameter, because transcription, translation, and localization in the case of proteins, or synthesis, packaging, and vesicle delivery in the case of wall components, are spatially localized on a much shorter length scale. Nevertheless, there is some evidence for long-distance protein translocation during fruiting in *Agaricus* (58).

In comparison with diffusive movement, motor-driven organelle transport can provide constant velocity transport in both directions over medium-length scales (millimeters to centimeters) both for the cargo transported, and also the bulk cytoplasm that becomes entrained (40). Fungi have some of the fastest microtubule motors known (1 to 4 μm s^{-1}) (59), while myosin V in yeast transports secretory vesicles at ~3 μm s^{-1} (44, 60). The maximum speed at which motors can move also places an upper bound on the velocity that can be achieved by motor-driven movement to approximately 4 μm s^{-1} (14.4 mm h^{-1}) (61). Actin-myosin-based cytoplasmic streaming in other systems, such as pollen tubes, can reach higher velocities, typically 40 to 60 μm s^{-1}, and reaching 100 μm s^{-1} with myosin XI in characean algae (62), but such high-speed motors do not seem to operate in fungi. The energetic costs of motor-driven movement would be expected to scale with the length of the transport pathway, particularly the cost of assembling the microfilament and/or microtubule tracks, the size of vesicles or organelles transported, the speed of movement, and the cytoplasmic viscosity (62). There are no quantitative estimates in fungi to our knowledge, but it is likely that the energetic costs are excessive, particularly in comparison with mass flow (see below). Nevertheless, this rate of movement may well be sufficient for species that do not forage far beyond their food resource, termed resource-restricted or non-translocators (57, 63), or for mycorrhizal fungi that have access to an abundant and consistent supply of carbon/energy from the host plant.

In addition to motor-driven transport, passive movement of solutes and organelles, such as vacuoles and nuclei toward the tip, can occur through mass flow of cytoplasm arising from a combination of expansion of the apical cell wall to create new volume, and subapical uptake of water and solutes (32, 33, 64–68), termed growth-induced mass flow (see Fig. 1B, 2B, and 3) (33). Quantitative microscopic measurements have been made on *Neurospora* hyphae, which have a relatively large diameter and grow at a maximum rate of 1 μm s^{-1} equivalent to 3.6 mm h^{-1} (69), thus capturing the fastest local transport events required for tip growth in any species. Injection of oil droplets, which cannot interact directly with motors and the cytoskeletal system, provides direct evidence for growth-induced mass flow, as the rate of movement (about 5 μm s^{-1}) exceeds the rate of tip extension (0.2 to 0.5 μm s^{-1}) in these experiments (66). The actual velocity expected for mass flow depends on the number of downstream tips (Fig. 2B; see "The impact of branching on transport" below). Likewise, nuclei, vacuoles, and mitochondria in apical compartments all migrate toward the growing tips at the same or greater rate than the tips extend (64, 66–68). Such movement would be consistent with mass flow driven by the continuous subapical water influx required to sustain volume increases at the tip during growth.

The time (t_A) taken for solutes or organelles to advect a distance l at velocity v is l/v, while the relative importance of advection compared with diffusion is given by the Péclet number, $t_A/t_D = vl/D_j$ (62). Advection dominates if this ratio is greater than 1, while diffusion dominates if this ratio is less than 1. Thus, the Péclet number for the apical compartment of *Neurospora* growing at 1 μm s^{-1} is approximately 0.3 for solutes and 20 for proteins. An alternative measure considers the length scale for localization of macromolecules, such as proteins and mRNA, depending on diffusion, advection, and their lifetime () before degradation (65). Thus, localization scales as $\sqrt{D_j}$ if diffusion and degradation dominate, v if flow and degradation dominate. The ratio $C = \sqrt{D_j/v^2}$ of these two distances is a dimensionless number. If $C > 1$, then most transport occurs by diffusion, and if $C < 1$, then most transport is by flow (65).

Water Permeability and Water Uptake

The extent to which longitudinal mass flow occurs is critically dependent on precisely where water influx

occurs in the colony. In principle, water could be taken up anywhere along the hyphae, but the actual site of uptake has profound implications for the magnitude and direction of internal mass flows. A second critical observation is that regardless of intrahyphal concentration gradients and turgor pressure resulting from solute accumulation, mass flow only takes place when water is able to exit the translocation pathway. A range of possible routes exists through either localized exudation (70–73), hydraulic redistribution to soil with lower water potential (74), evaporation during fruit body formation (e.g., *Phycomyces* sporangia) (75), coupling to the plant evapotranspiration stream in mycorrhizas (76), or by moving into a region of new growth (33, 66).

Fungi have a particular challenge to maintain a balanced water economy to allow water uptake for growth, while preventing excess evaporation from their extensive surface area. Equally, controlled evaporation may drive solute movement into aerial hyphae (75) and evaporative cooling may be required to aid spore dispersal by establishing convection currents (77), or through formation of "Buller's drop" by condensation (78). The plasma membrane provides some impedance to water movement, with permeability dependent on the lipid composition and temperature (79). Thus the osmotic permeability (P_f) of the plasma membrane in yeast mutants lacking aquaporins (AQPs) increases from 0.34 μm s^{-1} at 7°C to 2 μm s^{-1} at 23°C (80), equivalent to a plasma membrane water hydraulic conductivity coefficient ($L_w^{pm} = P_f V_w / RT$) of ~2.5 to 15 × 10^{-15} m s^{-1} Pa^{-1}. It is worth noting that these values are 1 to 2 orders of magnitude lower than plant and mammalian cell membranes at the same temperature. Indeed, the presence of ergosterol in fungi, rather than cholesterol in other organisms, may be linked to greater resistance to desiccation (81, 82).

The presence of AQPs (Fig. 1B) also significantly increases water permeability in parallel to the lipid bilayer. For example, overexpression of AQPs in yeast increased P_f by 9-fold at 7°C. Likewise, expression of AQPs from the filamentous mycorrhizal fungus *Laccaria bicolor* in *Xenopus* oocytes increased the membrane permeability to up to 147 μm s^{-1} (83). Whereas the precise role of AQPs in water uptake has not been resolved, AQPs do appear to be critical in hydraulic coupling during fruiting and between mycorrhizal fungi and the host plant (84–86).

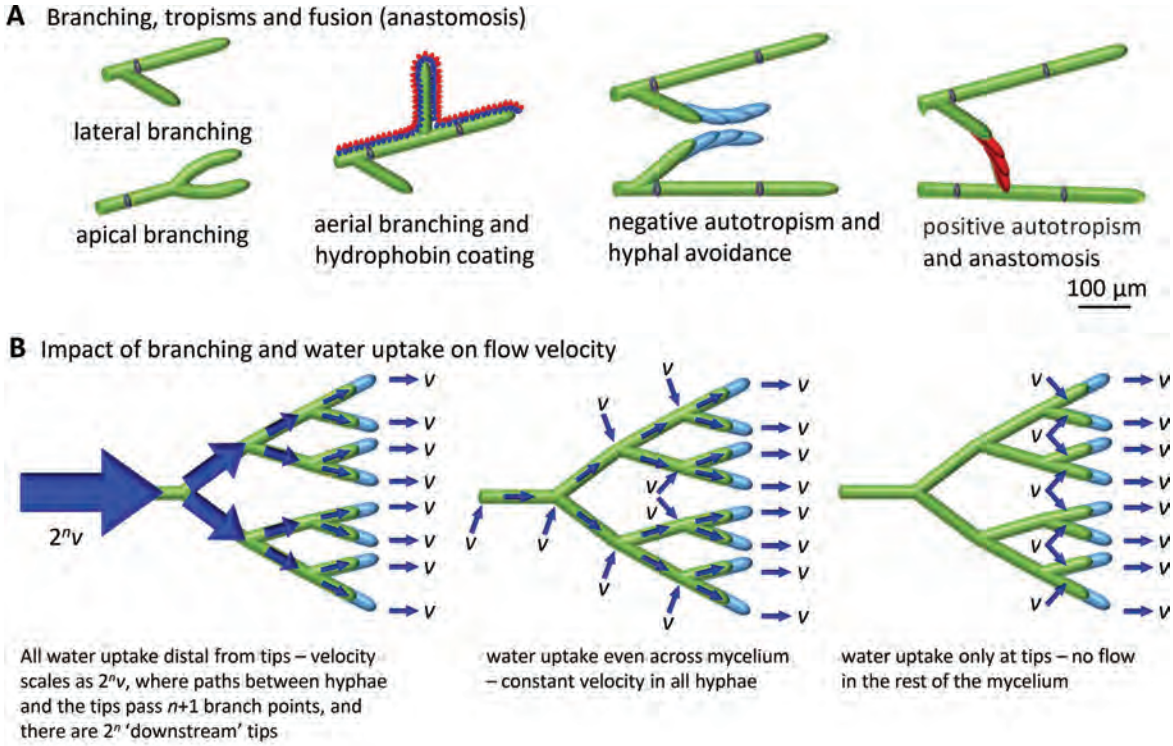
In both plant and animal systems there is also evidence for movement of water against the free energy gradient by chloride/cation cotransporters (CCC) (87, 88). CCC sequences are present in fungal genomes, but their role in fungal water economy has not been investigated to date. Nevertheless, this presents the interesting possibility that the low intrinsic plasma membrane water permeability in fungi, particularly at temperatures typically encountered in soils, might allow much greater regulated spatial control of the water economy through the localization and activation of aquaporins and CCC water pumps.

Hydrophobins and Control of Water Loss

To restrict water loss, many mycelia produce small (15 kDa) secreted cysteine-rich hydrophobin proteins that self-assemble to form a rodlet layer, particularly on aerial hyphae (Fig. 1B and 2A), and increase the hydrophobicity of the surface, thus restricting water movement (89–91). One benefit of using hydrophobins to reduce evaporation is that their intrinsic organization varies with water availability, with no additional sensing or control other than their synthesis and secretion, allowing them to function autonomously outside the cell. Nevertheless, hydrophobins represent an expensive investment of scarce nitrogen resources, particularly as the cost of forming a functional barrier scales with the surface area of the hyphae covered. Thus, in more mature systems and multihyphal aggregates, hyphal walls tend to be impregnated with other compounds, such as melanin and phenolics, to reduce water evaporation and act as protection against damage and fungivores (92). Species vary in the extent of hyphal hydrophobicity, and this characteristic has been used to group mycorrhizal species as hydrophobic or hydrophilic (73).

In addition to chemical impregnation, there are biophysical constraints on water evaporation through the wall interstices depending on the diameter of pores in the cell walls generating a matric potential. There are few measurements of pore size in fungi, but in the water mould *Achlya* a value of 2 nm was determined experimentally (93). If terrestrial fungi have similar values, this would restrict evaporation, even at low external relative humidity, depending on the radius of the pores and wettability of the walls (79). While the role of matric potentials is well understood for the soil-plant-atmosphere continuum (79), we are only at the beginning of understanding water fluxes through the more complex soil-mycorrhiza-plant-atmosphere or soil-saprotroph-atmosphere systems (76, 94).

Insulating the hyphal wall leads to secondary problems on how the fungus can then sense the external chemical environment, how enzymes needed to decompose organic resources can be delivered, and how the products are then absorbed. Interestingly, there is evidence that enzyme delivery may be mediated by


A Branching, tropisms and fusion (anastomosis)
lateral branching
apical branching
aerial branching and hydrophobin coating
negative autotropism and hyphal avoidance
positive autotropism and anastomosis
100 µm
B Impact of branching and water uptake on flow velocity
2ⁿv
All water uptake distal from tips – velocity scales as 2ⁿv, where paths between hyphae and the tips pass n+1 branch points, and there are 2ⁿ 'downstream' tips
water uptake even across mycelium – constant velocity in all hyphae
water uptake only at tips – no flow in the rest of the mycelium
C Formation of multi-hyphal aggregates
Formation of tendril hyphae
tendril hyphae
0.5 mm
Loose strand formation
tendril hyphae
0.5 mm
Cord formation and vessel differentiation
fibre hyphae
tendril hyphae
vessel hyphae
0.5 mm
Multi-hyphal rhizomorph formation
rind
cortex
medulla
lacuna
2 mm
apical cap
D Long-distance translocation in multi-hyphal aggregates
Cytoplasmic streaming allowing both acropetal and basipetal movement of nutrients
net acropetal mass flow in vessel hyphae in response to growth

unconventional secretion through extracellular vesicles (EVs, exosomes), possibly derived from multivesicular bodies, that cross the wall (Fig. 1A) by an unknown mechanism (43, 95).

The Impact of Septation on Transport

In Dikarya, after a period of growth, the apical cell divides to establish a series of septal compartments that retain cytoplasmic continuity through the septal pores (Fig. 1C). Septa exhibit a variety of pore structures from narrow plasmodesmatal connections or simple uniperforate pores, to pores with associated Woronin bodies or other pore-occluding structures in the Ascomycota, and the more complex septal pore caps in the Basidiomycota (Fig. 1C) (96). The pore size ranges from 10 to 70 nm in the case of plasmodesmata and from 50 to 500 nm for most other pore types but is often partially occluded by electron-dense material (6, 96). Septal pores allow the passage of nutrients, macromolecules, and organelles between septal compartments. In some species, nuclei can also migrate through the septal pore (67, 69), while in the basidiomycetes, nuclear movement in heterokaryons is strictly controlled through the formation of clamp connections adjacent to the septum. The pore may be blocked reversibly or irreversibly by Woronin bodies (WBs) and septal pore associated (SPA) proteins in ascomycetes, or the septal pore cap (SPC) and its various elaborations in basidiomycetes (6, 97). Interestingly, the precise positioning and tethering of WBs have recently diverged in the Pezizomycotina, with more distal anchorage in rapidly growing species such as *Neurospora* (Fig. 1C), regarded as an adaptation to prevent accidental pore occlusion as high flow velocities sweep the WBs into the pore (98). While pore occlusion has a role in preventing loss of cytoplasmic contents after damage (6), there is increasing evidence that pore closure (99) or opening (100) can occur as part of a developmental sequence, and closure may be reversible depending on environmental conditions (101).

Even when open, the septal pore places an interesting constriction on fluid flow between septal compartments (Fig. 1D). The velocity of flow through septal pores (v_p) increases in proportion to the relative area of the pore to the cross-sectional area of the hypha (r_h^2/r_p^2), with experimentally measured velocities in excess of 200 μm s^{-1} in *Neurospora* (69), sufficient to restrict backward diffusion against the flow (65). Various organelles also accumulate in local eddies adjacent to the septa and, in the case of nuclei, appear to alter their protein complement as a result (69). Indeed, accumulation of secretory vesicles at septa may also be part of the trigger initiating branch formation (102, 103).

Fluid flows induce wall shear stresses, and in the case of laminar flow, the wall shear stress τ produced can be estimated using the formula $\tau = 4\eta v/r$, where η is the dynamic viscosity of the fluid, v is the mean velocity of fluid flow, and r is the radius of the vessel (104). In the typical case where the fungal cytoplasm has dynamic viscosity of 2 g s^{-1} m^{-1} and the hypha has a radius of 6 μm, the wall sheer stress is $\tau = v \times 10^{-3}$, where τ is measured in Pascals and v is measured in μm s^{-1}. By way of comparison, the wall shear stresses in mammalian arterial systems are in the range of 0.2 to 2 Pa, and it is known that stresses of that scale induce changes in gene expression (105). Hence, it is plausible that a 6-μm-radius hypha could detect and respond to internal velocities of the order of 1 mm s^{-1}, but along the length of most hyphae the wall shear stress will be negligible. However, because the mean velocity of fluid flow is inversely proportional to cross-sectional area, and wall shear stress is proportional to the velocity

Figure 2 Mycelial network formation: branching, fusion, and multihyphal aggregate formation. (**A**) Hyphae may branch subapically or by tip splitting to explore the substrate or to form aerial hyphae that are often insulated by hydrophobins. At the colony margin, tips avoid each other, while secondary branch hyphae in the colony interior can show positive autotropism and fuse by anastomosis. (**B**) The velocity of mass flow within the network depends critically on the site of water uptake for growth. If all water uptake is distal from the tips, the velocity scales in proportion to the number of downstream tips. If uptake is equal everywhere, the flow rate is constant at the speed of hyphal extension, while if uptake is solely at the tips, there is no long-distance movement. Based on references 32 and 33. (**C**) Schematic representation of the formation of strands, cords, and rhizomorphs, showing progressive differentiation of vessel hyphae as potential conduits for long-distance transport. Scale bars are approximate. Based on references 127, 129, 134, 228, and 265. (**D**) Schematic representation of how circulating fluid flows might operate within a hyphal cord. Acropetal mass flows would take place in the vessel hyphae in response to growth, evaporation, or exudation at the tips, while basipetal flows take place simultaneously through cytoplasmic hyphae by cytoplasmic streaming. Redrawn from reference 17 with permission.

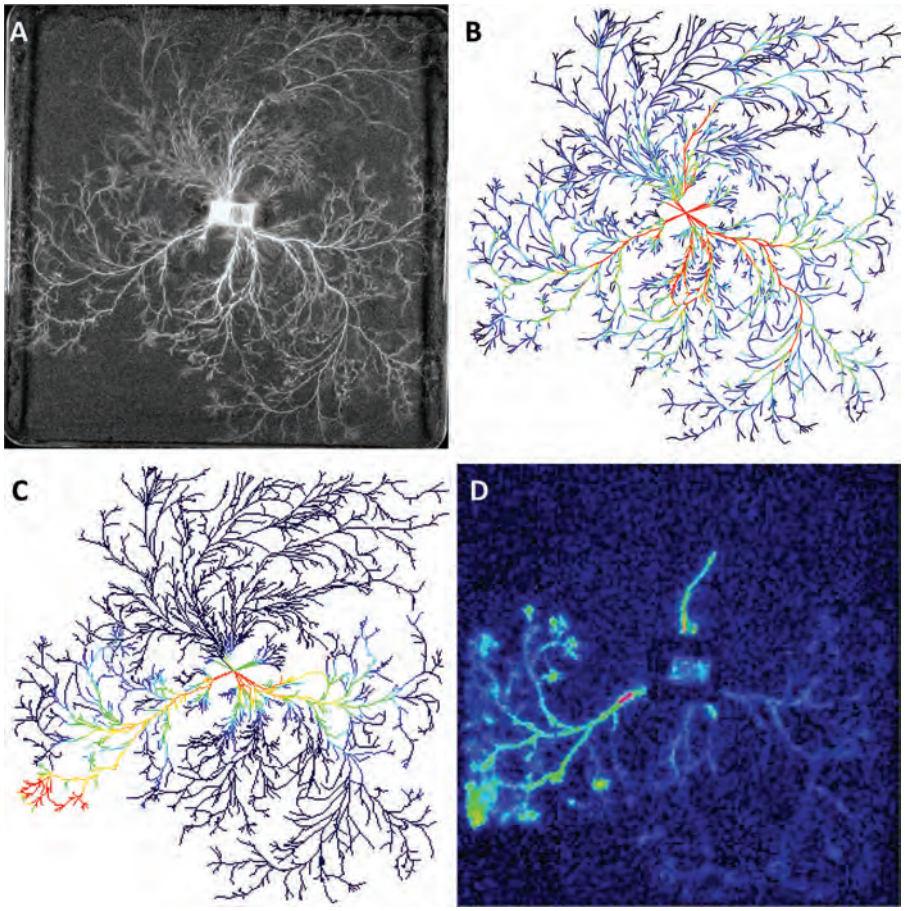


Figure 3 Growth-induced mass flows explain long-distance nutrient movement in *Phanerochaete velutina*. (**A**) Structure of the mycelial network after 21 days growing from a wood resource across compressed black sand. (**B**) The network architecture is extracted using intensity-independent, phase-congruency tensors and watershed segmentation from experimental time series. The output is a set of weighted adjacency matrices of the length, width, and volume of each cord. In this image, the cord width is pseudo-color coded from 50 μm (blue) to 500 μm (red). (**C**) The network structure and growth are input into a biophysical advection/diffusion/delivery (ADD) model, using growth-induced mass flow to predict the pattern of resource translocation. The predicted amount of radiolabel is color coded on a rainbow scale from blue (zero) to red (maximum). (**D**) The actual pattern of nutrient movement is then determined experimentally by using the nonmetabolized amino acid analogue ^{14}C-amino isobutyrate (^{14}C-AIB) and photon-counting scintillation imaging (PCSI). The amount of ^{14}C-AIB is color coded on a rainbow scale from blue (low) to red (high). The ADD model of growth-induced mass flow predicts the distribution of radiotracer in a complex network of fungal cords with a Pearson correlation coefficient 0.56. From Heaton et al. (32) with permission.

gradient at the wall, wall shear stress at the septal pore will be approximately r_h^3/r_p^3 greater than the wall shear stress elsewhere (where r_h and r_p are the radii of the hyphae and pore, respectively). This means that even the modest flows that have been measured in fungi can be expected to produce physiologically significant mechanical forces at the septal pore. Indeed, the scale of those forces can be estimated by measuring the elasticity of the cell wall and the amount of deflection that occurs during transient pore plugging, and in *Neurospora crassa* it has been calculated that the deflection-associated pressure difference is as large as 2 bar (69). Also note that the above equations somewhat underestimate wall shear stress, because the velocity profile deviates from the parabolic profile expected from Poiseuille flow due to the high density of organelles

moving within the fluid (68). This analysis suggests that septal dissolution, branching, and other aspects of hyphal development may be directly influenced by the relative scale of flows within the hyphae (33), because the mechanical forces are large enough to degrade septa (69), and it is also plausible that fluid flows could be detected by proteins located at the septal pore, leading to flow-dependent changes in the pattern of gene expression.

Modeling Tip Growth and Branching

The number of growing tips at the colony margin increases by apical or subapical branching and daughter hyphae typically show avoidance behavior (negative autotropism, Fig. 2A) regulated by the Cdc42 signaling pathway (7, 106) to increase exploration of the substrate (Fig. 2A). The fact that fungi grow by apical extension, although intercalary growth has been observed (107, 108), implies that there is a fundamental relationship between the overall colony growth rate and the branching rate. This relationship can be clarified by letting A denote the mean cross-sectional area of individual hyphae, $N(t)$ denotes the number of hyphal tips at time t, $g(t)$ denotes the specific growth rate of the colony (that is, the rate of change of volume per unit volume of fungus), and $v(t)$ denotes the mean velocity of hyphal tips. If all growth is due to apical extension, it follows that the mean volumetric growth rate of an individual tip is $Av(t)$, and the total volumetric rate of growth $\frac{dV}{dt} = V(t)g(t) = AN(t)v(t)$. It follows that:

$$N(t) = \frac{V(t)g(t)}{Av(t)} \quad (1)$$

When the specific growth rate g and the mean velocity of hyphal tips v are constant, the rate of change of the number of tips is:

$$\frac{dN}{dt} = \frac{\frac{dV}{dt}g}{Av} \quad (2)$$

Equations 1 and 2 imply that when the specific growth rate g and velocity of hyphal tips v are constant, we have:

$$\frac{\frac{dN}{dT}}{N} = \frac{\frac{dV}{dt}}{V} = g \quad (3)$$

Note that the number of tips is increased by each branching event and decreased by each fusion (anastomosis) event, so we cannot conclude that the branching rate is simply equal to the specific growth rate g. Nevertheless, it is reasonable to assume that the branching rate is only slightly larger than the specific growth rate g, which is a claim that has empirical support (102, 109). If the branching rate is equal to the specific growth rate, there must be a constant relationship between the volume of hyphae and the number of hyphal tips. In 1959, Plomley (110) observed that exponential colony growth requires continual branching, and he speculated that when a species is growing exponentially, there is a constant volume of fungus per hyphal tip termed the "hyphal growth unit." This is the key concept behind the mathematical models of hyphal growth developed in the 1970s (102, 109, 111–113), which recapitulate the experimental observation that the velocity of hyphal tips is initially proportional to the volume per hyphal tip (e.g., reference 69). Thus, the rate of germ tube extension is initially exponential as the increasing volume of cytoplasm provides more vesicles per tip. As the colony grows, the rate of vesicle delivery reaches a maximum and the mean tip velocity approaches a constant as a result, obtaining a value for the hyphal growth unit that is characteristic of the species in the given environment and manifest at a colony level as the width of the peripheral growth zone (111).

Linking Growth and Branching to Nutrient Status

Like all organisms, fungi require a source of energy and nutrients to sustain growth, and so the specific growth rate and branching rate of fungi must be smaller for fungi that grow on substrates that are recalcitrant or nutrient poor (114, 115). This kind of energetic constraint on fungal growth has implications for the optimal allocation of resources toward vegetative growth, the production of digestive enzymes, and the production of spores (31). Any successful model of hyphal growth must recapitulate the fact that nutrient availability enables growth, and rapid growth is associated with rapid branching. However, because of the difficulty of observing fungal growth and a lack of understanding of the control of branching, there are few statistics concerning the location of branching events in a growing fungus (116). On a macro scale, models of the branching process generally make the reasonable assumption that branching is more likely to occur in regions with a higher density of tip-growth vesicles (29, 102), which may occur when vesicles are too far downstream of the apex to be able to contribute to growth of the apex itself and are therefore diverted to form a new growing tip (102, 117). On a micro scale, it has been suggested that branching is associated with septa because vesicles may accumulate around septal pores (102, 103, 109, 117). However, it is not known wheth-

er, all else being equal, younger hyphae are more likely to branch, or whether hyphae that carry relatively large mass flows are more likely to branch.

Hyphae can also form in older parts of the colony by secondary or lateral branching, to an extent dependent on the species, the medium of growth, and other environmental factors (8, 118, 119), but there are significant differences in the cellular mechanisms involved in apical and secondary tip formation (120). Watters et al. (121) demonstrated that the distribution of distances between adjacent branch points is independent of tip extension rate in *N. crassa* at a range of temperatures. However, following a sudden increase in temperature (and metabolic rate), there is a transient increase in branching rate, while, following a sudden decrease in temperature, hyphae produce an unusually long branch interval (122). These observations are consistent with the following four assumptions:

1. The rate of tip extension is proportional to the rate of vesicle deposition.
2. The rate of branching is proportional to the number of vesicles in the colony.
3. The rate of vesicle production is proportional to the metabolic rate of the colony, and this rate changes relatively rapidly when the metabolic rate is altered by changing temperature.
4. The rate of vesicle deposition at the tips is proportional to the metabolic rate of the colony, and this rate changes relatively slowly when the metabolic rate is altered by changing temperature.

If these plausible assumptions are correct, a sudden increase in temperature will increase the rate of vesicle production more quickly than it increases the rate of vesicle deposition, creating a transient increase in the density of vesicles within the colony, which produces an increase in the branching rate. Also note that, if the velocity of hyphal tips and the specific growth rate of a fungus remain fairly constant, the diameter of the fungal colonies will grow in a linear manner, while the volume of that same colony will grow exponentially. This implies that the density of the fungal growth will increase, although at some point population effects will start to inhibit growth because, in the dense core of the colony, the concentration of inhibitory waste products will be relatively high and there will be relatively few nutrients or space to enable further growth.

The Impact of Branching on Transport

Branching reduces the radial distance that can be supplied by diffusion alone (53), but has no effect on the velocity of motor-driven transport, although the total flux is halved at each branch point. The extent to which branching affects organelle and resource distribution by mass flow depends on the site of water uptake (16, 32, 33). At one extreme, if water uptake only occurs at the initial inoculum, volumetric mass flow scales with the number of downstream tips that are growing (Fig. 2B). If water uptake takes place equally across the network and the cross-sectional area is constant, the velocity in each hypha matches the rate of growth, while, if all uptake takes place at the tips, there is no mass flow in the rest of the colony. In wild-type or the *so* mutant of *Neurospora*, which is unable to fuse and thus only grows as a branching tree (123), nuclear movement occurs predominantly by mass flow, and the rate scales with the number of downstream tips, reaching values of 5 to 20 $\mu m\ s^{-1}$ (67, 69), consistent with water uptake at the center of the colony or germinating conidium. Thus, in *Neurospora*, even though water is freely available in the substrate, uptake is highly spatially regulated to ensure high rates of longitudinal flow through the colony.

The Impact of Hyphal Fusion on Transport

Fusion may take place early in colony formation *via* conidial anastomosis tubes (CATs) that potentially link multiple conidia into a supracellular network (11–13). Secondary branch hyphae also develop in more mature regions when daughter hyphae typically show positive autotropism and fuse with neighbors to create a microhydraulic network with loops (7, 9, 11–13, 49). Fusion appears to be required for high rates of long-distance nutrient translocation, as the *so* mutant in *Neurospora* shows reduced radiolabeled amino acid movement (124). Nevertheless, loops are probably more critical in providing multiple pathways to improve network resilience and to allow rapid reallocation of resources in response to local demands. For example, lateral movement of radiotracers at the colony margin (125) probably occurs as a result of anastomosis.

Hyphal Differentiation and the Formation of Multihyphal Aggregates

In many species, there is some differentiation into leader or trunk hyphae and aerial hyphae at the colony margin (Fig. 2C). However, formation of linear hyphal aggregates, such as cords and rhizomorphs, only occurs to a lesser extent on agar, so there is comparatively little information on the development and internal structure of these organs from laboratory studies.

Cords form behind the front of apically extending hyphae, with the degree of hyphal aggregation within the growing front varying between species (126, 127).

Close to the growing front, both large- and smaller-diameter hyphae are normally present with many branches and anastomoses interconnecting both hyphal types. In corded systems, large-diameter hyphae are initially cytoplasmic, with progressive cellular disorganization and appearance of empty vessel hyphae with distance from the front (17, 128). In *Serpula lacrymans* narrow tendril hyphae emerge from the clamp connections and then branch and grow both acropetally and basipetally around neighboring hyphae (129). In more developed cords, cross sections at the light or EM level show further differentiation of thick-walled fiber hyphae and vessel hyphae, with diameters of approximately 10 to 15 μm, that are likely to improve flow (Fig. 2C). During early development, movement of radiotracers and fluorescent dyes appears to be restricted to particular routes that probably correspond to developing cords (125, 130–132), although tracking the internal connectivity has been rarely attempted. The size of the vessel hyphae can have an impact on the flow rate, which scales as r^4, but flow will be more critically dependent on the size of the septal pores and the extent of septal dissolution associated with cord formation (126, 127). Nevertheless, ectomycorrhizal cords of *Paxillus* have swollen clamp connections at the nodes that provide continuity with the branches, but only limited septal dissolution in the vessel hyphae (133).

Rhizomorphs are more highly differentiated root-like structures that extend from a meristematic-like region at the tip (Fig. 2C). They consist of a central lacuna surrounded by an inner medulla with large thin-walled vessel hyphae that form high-conductivity channels, an outer medulla containing loosely packed hyphae that are only about 2 μm wide, and a thick melanized, hydrophobic rind that insulates them from the environment. The longitudinal hydraulic conductivity ($L_w^{\parallel}$) through the cords varies from 1.5 to 3×10^2 cm^2 bar^{-1} s^{-1} (134). The hydraulic conductivity perpendicular to the hyphal axis ($L_w^{\perp}$) for such cords has not been measured, but is likely to be low.

Pressure-Driven Mass Flow on a Macroscopic Scale

The velocity of water movement in corded systems or rhizomorphs is vastly in excess of values that could be achieved by motor-driven movement, and initially led to the idea of pressure-driven mass flow as the major transport mechanism in foraging fungi (15, 135). Thus, estimates of water transport in *Suillus bovinus* were 27 cm h^{-1} (136), while measurements in *S. lacrymans* have a mean value of 148 cm h^{-1} (70). Likewise, a range of different solutes is transported in *Armillaria* at velocities in excess of 20 mm h^{-1} (137), in *Serpula* ranging from 10 to 50 mm h^{-1} (138) to 20 to 30 cm h^{-1} (135, 139), or 20 to 100 mm h^{-1} in *P. velutina* depending on the extent of cord formation (32, 33, 131, 132; Fricker et al., unpublished data). Similarly, even movement of larger organelles, such as nuclei, by mass flow can reach velocities of up to 4 mm h^{-1}, although there is wide variation in velocity between different hyphae in the network (67).

The magnitude of growth-induced mass flows has been empirically determined by measurements of network growth at the level of individual cords (Fig. 3A and B) (33), and input into an advection-diffusion-delivery (ADD) model to predict the pattern of resource translocation (Fig. 3C) (32). The actual pattern of nutrient movement was then determined experimentally using non-metabolized radiotracers and photon-counting scintillation imaging (PCSI; Fig. 3D). The ADD model of growth-induced mass flow is surprisingly effective at predicting the distribution of the amino acid analogue ^{14}C-amino isobutyrate (^{14}C-AIB) in a complex network of fungal cords (compare Fig. 3C with Fig. 3D). This is remarkable considering the conceptual simplicity of the biophysical model, and that there is essentially only one free parameter, the fraction of the cord that carries nutrient flow.

The magnitude and direction of mass flows can be altered by experimental manipulation of the external water potential, but it is also clear that, under natural conditions, the external water potential can have an impact on transport through the network. Thus, substantial water flow (18 cm h^{-1}) can be driven by hydraulic redistribution from moist to dry soil (74), or through hydraulic coupling to the host plant (27 cm h^{-1}) in the case of mycorrhizas (136, 140).

Biomass Recycling

As some hyphae expand and mature, other regions of the mycelium regress and their contents are recycled. In particular, there is extensive remodeling of redundant parts of the mycelium in larger mycelia grown from woody resources (see "Colony dynamics and behavior" below), presumably to redeploy resources to the growing margin to forage more efficiently in highly heterogeneous environments (14, 31, 56, 141, 142). Interestingly, in other systems, such as blood vascular networks and slime molds, recycling and network remodeling occur in response to flow rate (143), and there is evidence that a similar relationship between high flow and cord thickening, versus low flow and cord thinning, holds for fungi (33, 144).

At a molecular level, recycling probably involves autophagy and controlled apoptotic-like mechanisms, rather than necrosis (145, 146). Nevertheless, how decisions are made to trigger autophagy in particular hyphae are not clear, nor is it clear how the cytoplasm and wall materials are broken down and transferred to the growing front.

Bidirectional Movement and Oscillations

There is plenty of evidence for nutrient translocation from resources to the growing margin where they are needed for growth. However, in several systems, radiotracer measurements indicate that nutrients can move both acropetally and basipetally in the same colony or following colony fusion (125, 137, 138, 147–152). Bidirectional movement is likely to be critical to achieve mixing of spatially distributed food resources between constantly changing sources and sinks and therefore promote maximum colony growth in a heterogeneous environment, and may also contribute to colony-wide control of behavior (56). Over short length scales, diffusion and motor-driven transport may achieve transport at a sufficient rate, but responsive resource allocation in larger colonies requires mass flow. However, simultaneous bidirectional movement is difficult to explain based solely on pressure-driven mass flows, which would be expected to operate in one direction at a time, so models have invoked distinct pathways that involve acropetal mass flows in vessel hyphae and basipetal streaming in adjacent cytoplasmic hyphae (Fig. 2D) (17). In addition, some species, such as *P. velutina* (131, 132, 153), but not others, such as *S. lacrymans* (138) or *N. crassa* (124), show marked oscillations superimposed on the net transport. The origins of the oscillations are not known, but may represent a reciprocating transfer of material, rather than the continuous circulatory system shown in Fig. 2D. This is reminiscent, albeit at a much longer time scale, of the shuttle streaming in *Physarum polycephalum* that leads to efficient mixing within the plasmodium (154).

COLONY DYNAMICS AND BEHAVIOR

In discrete organic resources saprotrophic mycelial networks typically operate on a millimeter to centimeter scale, depending on the size of the resource and size of the genetic individuals. However, large individuals occupying extensive decay columns in tree trunks and branches operate over a larger scale. Furthermore, those fungi that are non-resource-unit-restricted extend from their main food base into the surrounding environment in search of new resources (Fig. 4 through 7), and often operate on the scale of meters. Thus, saprotrophic systems of mycelia cords and rhizomorphs cover several square meters to many hectares (Fig. 4) (135, 155, 156, 157). Currently, the largest recorded genet is of *Armillaria ostoyae* spanning 965 hectares, with a maximum separation of extending fronts of 3,810 m (157), although the extent of internal connectivity is unknown.

There is little information on mycelia networks within organic resources (see "Extension of network analysis to 3-D" below), except for the boundaries of territory occupied by individual mycelia, indicated by interaction zone lines (36). Likewise, little is known of the structure of networks of non-resource-unit-restricted fungi foraging in relatively homogeneous resources, e.g., fairy ring formers and colonizers of leaf litter patches (36). The latter are particularly interesting because they extend through the leaf litter layer as an ever-increasing annulus of mycelium, about 30 to 40 cm wide in the case of *Clitocybe nebularis*, differentiated into several distinct zones (158).

A wide variety of patterns of mycelial outgrowth from organic resources has evolved, ranging between slowly extending search fronts densely populated by hyphae, which are unlikely to miss new resources, e.g., *Hypholoma fasciculare*, to more rapidly extending but sparser systems, e.g., *P. velutina*, and at the furthest extreme *Armillaria* species. The slow-dense foragers are often considered short-range foragers, and have sometimes been termed "phalanx or phalangeal foragers" (34, 35, 159), by analogy with foraging of clonal plants that grow relatively slowly and have tight aggregated rosettes (160–163). The fast-effuse foraging, on the other hand, is often considered long-range foraging, and has been likened to "guerrilla" foraging of plants (clones of which have fast-growing branches that are loosely aggregated). Similar concepts have been developed for mycorrhizal networks that include short-range "contact exploration types" and "long-distance exploration types," where the mycelium is highly differentiated into strands (164). Other similar, but not identical, ideas have been put forward by plant ecologists, such as "clump" plants characterized by frequent branching at large angles and with short spacers, which exploit resources at a particular site, and "runners" with infrequent branching and long spacers, which explore more widely for resources (165, 166). Parallels have also been drawn between fungal and animal foraging (163, 167).

Perhaps the most dramatic alterations in mycelial network structure occur when new resources are discovered, either by a foraging mycelial front, or when

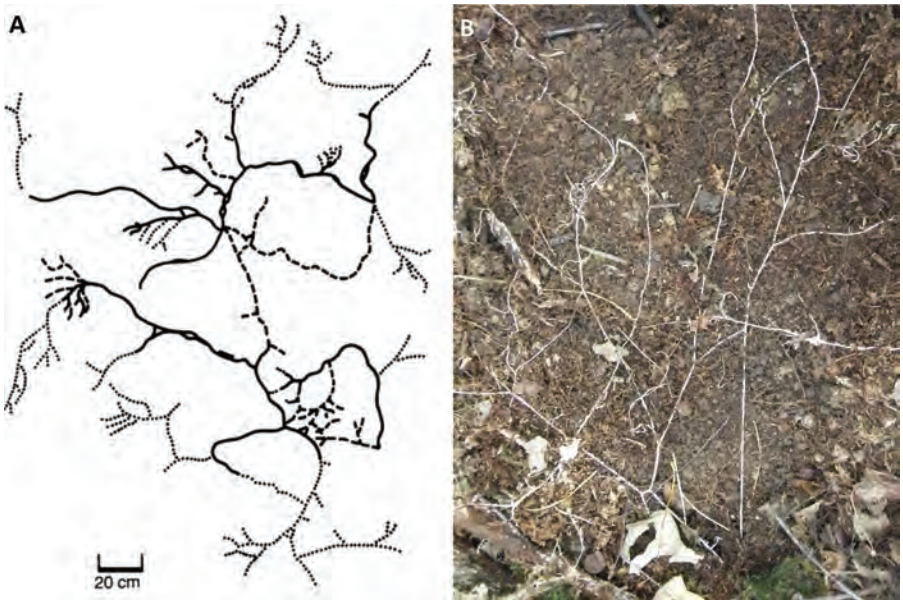


Figure 4 Mycelial networks in woodland. (**A**) Map of a mycelial cord network of *Phanerochaete velutina* revealed by carefully removing surface litter layer, recovering, and then rerevealing 13 months later. From Thompson and Rayner (155) with permission. (**B**) Network of *Megacollybia platyphylla* on the floor of a mixed deciduous woodland, following removal of surface litter.

resources land on an established system (Fig. 5). When mycelium encounters a new resource, parts of the network connecting the original with the new resource aggregate to form mycelial cords, while nonconnecting mycelium dies back (168). The rapidity and extent to which this occurs depends, among others, on the foraging strategy of the fungus, the relative size/quality of the original and new resources, and the presence/absence of invertebrate grazers (34, 35, 169, 170). Short-range foragers reallocate their biomass rapidly and extensively, with often only the connective cord and outward growth from the newly colonized resource remaining after a few months. In contrast, little reallocation of mycelial biomass occurs in long-range foragers unless the newly encountered resource is considerably more substantial than the original resource. The process is speeded up if grazing invertebrates are present; fine mycelium disappears more rapidly than if they are absent, leaving only larger mycelial cords (Fig. 5). These processes occur repeatedly on the forest floor, producing large, open networks of cords interconnecting discrete organic resources ranging from small twigs to large tree trunks (Fig. 4). How the decision is made to strengthen or recycle a particular cord is not clear, nor is the mechanism that serves to retrieve useful breakdown products and redirect them to regions of new growth.

Interestingly, strands of *Pleurotus ostreatus* and rhizomorphs of *Armillaria gallica* (formerly *bulbosa*) both show spontaneous action potentials between 0.5 and 5 Hz, and the frequency increases in response to addition of nutrients (171, 172). Furthermore, the signal propagated across the colony at a speed of 0.5 mm s^{-1} over distances of a few centimeters when nutrients were added remotely. Similar potentials have been reported in a number of other species, and it has been proposed that they may play a role in coordinating growth and differentiation by preventing hyphae from undergoing apoptosis and strengthening cord formation (172).

QUANTITATIVE ANALYSIS OF NETWORK ARCHITECTURE

Constraining the Problem to 2-D

Our understanding of the coordinated growth and behavior of fungal networks has been limited by the immense practical difficulties associated with measuring organisms that exhibit indeterminate, highly plastic growth. Although fungi would normally grow as three-dimensional networks through opaque media such as soil, wood, plant, or animal tissue, most morphological studies have constrained growth to two spatial dimen-

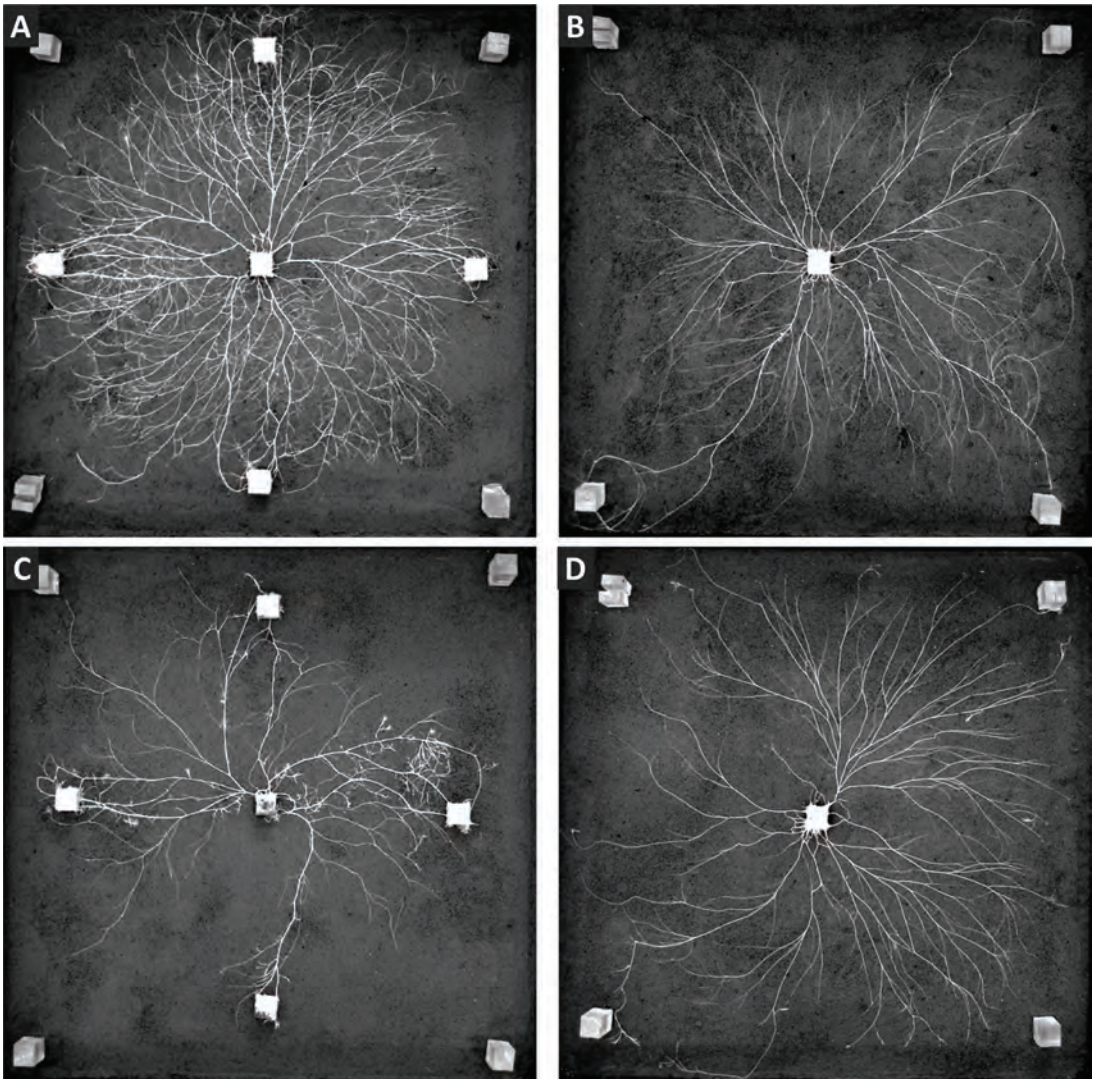


Figure 5 Effects of resource addition and grazing on mycelial networks. Mycelial cord systems (99 days old) of *Phanerochaete velutina* growing from centrally positioned beech wood inocula across the surface of compressed nonsterile soil in 50 × 50 cm trays. (**A**) With four additional beech wood blocks added behind the mycelial margin 36 days after central inoculation. (**B**) With no additional resources added. (**C**) As in A, but with 250 laboratory-reared collembola, *Folsomia candida*, added 49 days after the central inoculums. (**D**) As in C, but with no additional resources. From Wood et al. (170), with permission.

sions to simplify image collection and analysis. Furthermore, the substrate is usually limited to transparent media such as agar plates (e.g., 173), often overlaid with cellophane membranes (174, 175), or as dispersed mycelia in submerged cultures (176–178). There is a challenge to achieve both sufficient magnification to resolve individual hyphae and sufficient scale to map the entire network. Magnifications typically range from 10 to 100× using microscopes (179, 180), but photographic enlargers (174, 175, 181) or flatbed scanners can also be used (173). Colonies can also be grown on cellulose nitrate membranes on agar (182) or soil (183), and visualized following fixation, staining, and rendering the membrane translucent with immersion oil to enhance contrast (182). Alternatively, colonies grown on soil can be repeatedly sampled and growth quantified by using an immunoblotting technique (184).

Network organization is relatively easy to observe at the growing margin of the colony on transparent media. However, submarginal growth is denser and

includes aerial hyphae, which makes it difficult to track individual hyphae and discern the pattern of branching and anastomosis. Thus, most network analysis is limited to a short period (<72 h) of surface growth over short distances (millimeters to centimeters) (e.g., 173), although confocal imaging can be used to analyze 3-D distribution of aerial and penetrative hyphae labeled with Congo Red (185, 186). More recently, confocal fluorescence imaging following wall labeling with calcofluor white has allowed quantitative measurements of early colony formation in 3-D over an extended period (17 days) and represents the first time the dynamics of critical parameters such as branching length and angle have been determined throughout a colony (116). At a more macroscopic scale (centimeters to meters), saprotrophic fungi have been studied the most because they are straightforward to culture from woody resources as a planar 2-D system on nonsterile compressed soil or sand that can be readily imaged (Fig. 4 through 7).

Fractal Measures and Hyphal Coverage

The simplest quantitative measure of colony behavior is surface area coverage from the number of pixels that make up a mycelial image. However, like many natural structures, mycelia are approximately fractal, and fractal geometry may be more appropriate to describe the systems (173, 187, 188). The fractal dimension quantifies the extent to which mycelia fill space relative to the size of the mycelial system. In two dimensions, e.g., the surface of soil, its value ranges between 1 (a line) and 2 (a filled surface). In three dimensions it could take an upper value of 3, although three-dimensional mycelial systems have rarely been investigated. Two types of fractal dimension have been used to provide different measurements of the branching/space filling of mycelia: the mass fractal dimension (D_M) and the surface (or border) fractal dimension (D_S, a subset of D_M), which only describes the edge of the mycelial system. Both are important because they allow a distinction to be made between systems where there are gaps inside (i.e., hyphae do not entirely fill the space), and those systems that have plane-filled interiors and are only fractal at their edges/borders.

Several methods are available for calculating fractal dimension, the most commonly used for mycelia in natural and seminatural (i.e., not agar) situations, e.g., mycelia growing across the surface of soil, is the box-counting technique (188). Effectively, a series of grids of square boxes of different sizes (i.e., different numbers of pixels in an image; 3 to 63 being appropriate for mycelia in small [24 × 24 cm] soil microcosms) is overlaid onto an image of the mycelium. The number of boxes intersecting pixels of the mycelium is recorded. The box-count fractal dimension is given by D in equation 4:

$$N(s) \approx cs^{-D} \quad (4)$$

where $N(s)$ is the total number of boxes having side length s that intersect the mycelium image; c is a constant. There are two types of boxes: interior boxes (i.e., those that contain only pixels of the mycelium) and border boxes (i.e., those that contain at least one mycelial pixel and contain or adjoin at least one nonmycelial, e.g., soil, pixel). So the total number of boxes, $N(s)$, is given by equation 5:

$$N(s) = N_{border}(s) + N_{interior}(s) \quad (5)$$

Regression of the linear part of a plot of log $N_{border}(s)$ against log s provides an estimate of D_S (equation 6):

$$\log N_{border(s)} = \log c - D_s \log s \quad (6)$$

Likewise, regression of the linear part of a plot of $N_{interior}(s)$ against log s provides an estimate of D_M. D can be estimated for entire mycelia, or for distinct areas of local interest by using software such as FracLab for MatLab (https://project.inria.fr/fraclab/) or FracLac for ImageJ (189).

Fractal dimension and hyphal coverage per unit area vary considerably between species, during mycelial development, depending on abiotic conditions, and are altered by interspecific interactions with other organisms (188). D_M of mycelia in small (24 × 24 cm) microcosms of compressed soil (nonsterile, i.e., similar to the natural environment) ranges from 1, for the linear rhizomorphs of fungi such as *Armillaria* spp. and *Megacollybia platyphylla*, to close to 2, for the densely packed mycelia of *H. fasciculare* and *Stropharia caerulea* (Fig. 6). However, even for the latter two species, D_M decreases with time and increasing size of the system, with networks becoming sparser as mycelial cords form and interstitial hyphae die back. These mycelia with, at least, initially high D_M have lower D_S, indicating that they are surface/border fractal systems, i.e., only the edge of the system is fractal. Others have similar D_M and D_S, indicating that the whole system is fractal. Those fungi that are mass fractal tend to extend more rapidly than those that are border fractal, implying that there is a balance between space filling and extension rate. Trade-offs when deploying mycelial biomass in search of new resources, and for other attributes such as resilience, are considered in more detail below.

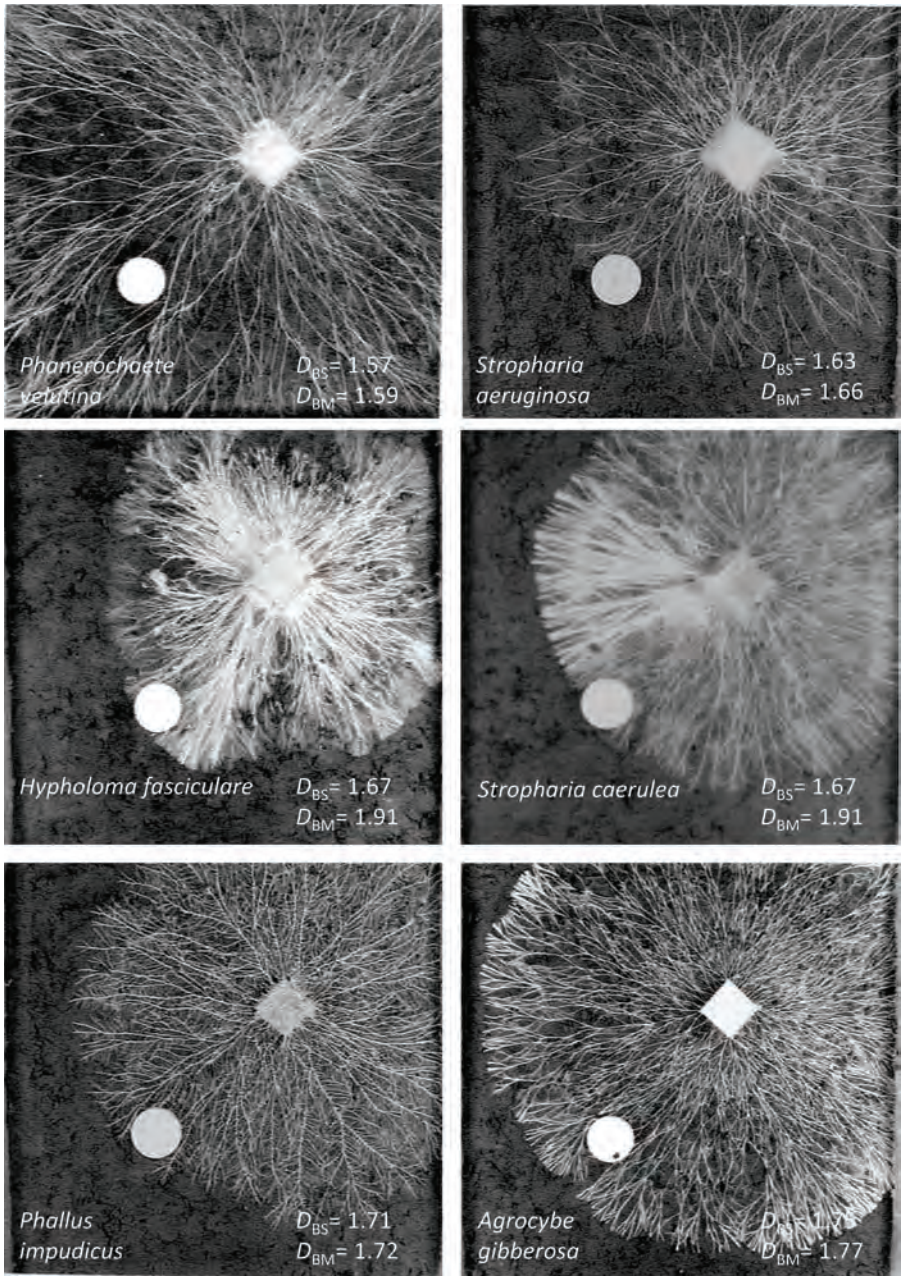


Figure 6 Species variation in fractal dimensions. Mycelia of six wood decay fungi extending, for 28 days, from centrally positioned beech wood inocula across the surface of compressed nonsterile soil in 24 x 24 cm trays. White circles are inert plastic discs. Note the different surface (D_{BS}) and mass (D_{BM}) fractal dimensions of the mycelia of different species. Photographs courtesy of Damian P. Donnelly. From reference 35 with permission.

Fractal geometry is affected by the quantity and quality of the resources available to the mycelium, including size of resource, number of resources, addition or encounter with new resources, and the nutrient status of soil through which the mycelium is growing (35, 187, 188). For example, D_{BM} and D_{BS} of *H. fasciculare*, *P. velutina*, and *Resinicium bicolor*, but not *S. caerulea*, increased with increasing inoculum size (from 0.5 to 4 cm^3). *S. caerulea* systems growing from well-decayed (84 days old) inocula took 10 days longer to achieve the same D_{BM} and D_{BS} values as when mycelia were growing from relatively undecayed (22 days

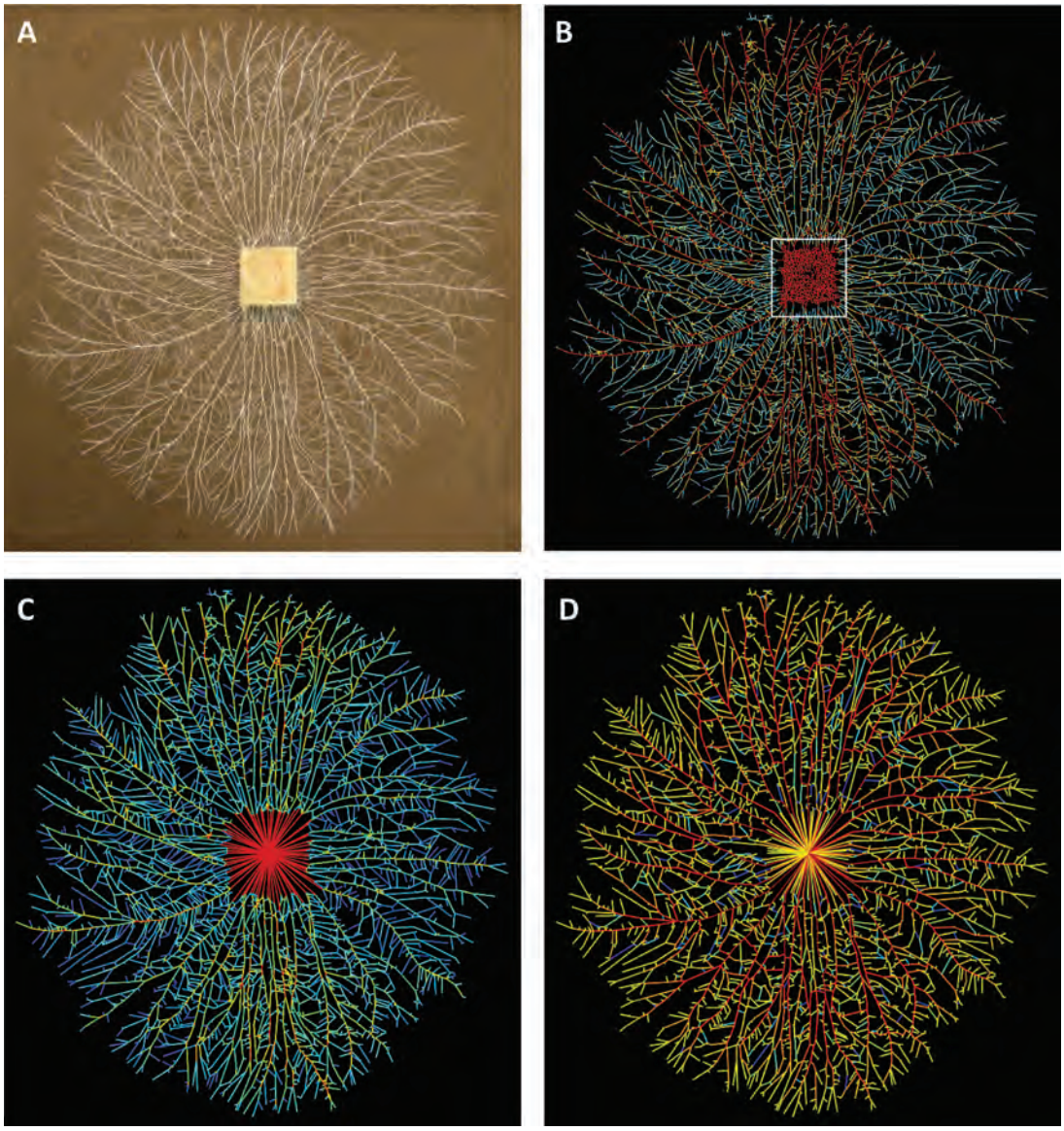


Figure 7 Macroscopic network analysis of *Phallus impudicus* growing on compressed soil. (**A**) Original image of the mycelial network after 21 days. (**B**) Network architecture automatically extracted using phase-congruency edge enhancement, watershed segmentation, and link pruning to give a single-pixel-wide skeleton pseudo-color coded by the cord width. (**C**) Conversion to a graph representation whereby each node (junction or tip) is connected by edges that are pseudo-color coded by the average width of the cord segment from 50 μm (blue) to 500 μm (red). The structure of the network within the wood resource cannot be defined so any cord incident on the boundary is connected to a central node with a link set to the maximum width. (**D**) Characterization of the cord betweenness centrality as a measure of how important each cord is to transport through the network from the resource to every other node, with the color code ranging from blue (low importance) to red (high importance).

old) inocula. D_{BM} and D_{BS} were often lower for mycelia growing across soils with relatively low phosphorous and nitrogen content, short-range foragers often being more responsive to nutrient addition than the longer-range foragers. Non-nutrient environment factors, including water potential, temperature, and pH, also sometimes affect D (188). For example, D_{BS} of *S. caerulea* decreased with increasing water potential

(i.e., getting wetter) from −0.02 MPa to −0.002 MPa, whereas with *P. velutina* there was little effect. D_{BM} and D_{BS} of *P. velutina* were significantly less at 5°C than at 10 to 25°C for several weeks, although the effects on *S. caerulea* were variable. Interactions with other fungi can also dramatically influence mycelium characteristics including hyphal coverage and fractal dimension, which varies between combinations of species and alters locally reflecting attack or defensive responses (190).

Graph-Theoretic Network Representations

Fractal measures capture some aspects of network organization, but advances in image analysis now mean that an explicit representation of the network structure is possible. Early image analysis routines (reviewed by references 176 and 177) used simple gray-scale thresholding (116, 180) or Sobel or Canny edge detectors followed by image opening and hole filling (191) to segment the hyphae. The resultant binary image is typically thinned to a single-pixel-wide skeleton and can be converted to a graph representation, where each junction is classed as a node and the intervening segment as a link. These approaches have the advantage that they can be readily implemented in open-source software packages, such as ImageJ (e.g., 182, 192).

More recent approaches use additional ridge enhancement algorithms, adaptive thresholding, and pruning steps to aid segmentation (173, 193, 194). Most current algorithms for network extraction were originally developed for blood vascular systems or neurons, and typically involve enhancement of the "ridge" nature of the hypha or hyphal aggregate. The most common approaches use second-order derivatives of a Gaussian over a range of angles and scales (e.g., 195), although pattern-matching templates can also be used (196). Anisotropic kernels are more powerful because they emphasize the elongated shape of network segments and have recently been developed specifically to analyze mycelial networks (197). The maximum response of the filter provides information on the edge strength and its orientation, while additional measures, such as the anisotropy of the first three eigenvalues, give a measure of how strongly the edge corresponds to a (blood) vessel ("vesselness," 195). An alternative approach uses local phase congruency to give an intensity-independent approach to edge enhancement. This provides better segmentation for larger microcosms with cords, when the edges vary in intensity as well as scale and orientation (Fig. 7A and B), but at the expense of greater computational cost. The phase-congruency measure can also be combined with tensor-based measurements to give the phase-congruency equivalent of "vesselness" (193, 198). The enhancement step is followed by segmentation using non-maximal suppression and hysteresis thresholding (197), watershed segmentation, and edge pruning (193), or a reverse diffusion-based algorithm (196) to give a single-pixel-wide skeleton.

A complete description of the mycelial network also requires an assumption of the hyphal width (e.g., 173), or experimental estimation from the network image (32, 33, 144, 169, 175, 193). This is still a technically challenging area with a number of methods available that use slightly different assumptions. For example, a granulometry approach can be used whereby the intensity image is subject to a series of image openings (erosion followed by dilation) that successively remove structures as the size of the opening kernel exceeds the underlying object (193, 198). The intensity of each pixel initially decreases slowly as the kernel samples more of the object, but then reduces dramatically once the boundary of the object is reached, and the kernel only samples the background. The transition point for any pixel is determined from the maximum (negative) gradient of the granulometry curve. However, this approach constrains the radius to integer pixel values, and also suffers from digital approximation of small kernels to a true disk-shaped kernel. Thus, rather than extract a specific size threshold, the integrated intensity under the granulometry curve can be calculated to provide a more nuanced interrogation of the local image intensity profile. The integrated intensity cannot be directly related to the physical width without additional assumptions about the relationship between intensity and sampled volume through calibrated microscopic measurements. Nevertheless, this approach does help with estimation of relative hyphal widths, even if they are subresolution objects, provided it is assumed that the intensity scales with the width of the hyphae (Fig. 7B). Including estimates of the hyphal width gives the volume of the mycelium at any point and, if time series are collected, how the volume changes with growth or recycling.

Conversion to a Graph Representation

The graph representation is based on translation of the pixel skeleton to a planar, weighted, undirected graph (Fig. 7C), with nodes located at hyphal tips, branches, and anastomoses, and edges representing hyphae or cords, with weights based on the Euclidean length (L) and radius (r) of each cord, combined either as the cylindrical volume ($V = r^2L$), to represent the material cost of the cord, or the predicted conductance ($G = r^2/L$), assuming cords are bundles of equally sized vessels

rather than a single vessel with increasing radius (144, 173, 175, 182, 199–201). The network cannot be resolved within the resource, so it is represented as a single node connected to all the edges incident on the resource boundary (Fig. 7C). The graph conversion step also makes additional assumptions about the functional connectivity of the network, because every junction or crossing point is automatically defined as a fully connected node in 2-D planar systems irrespective of whether there is an actual anastomosis present, although 3-D imaging can help to separate fusions from overlaps (116). In addition, the inferred conductivity assumes all the septal pores are open and hyphae behave as simple pipes.

Summary statistics, such as the number of tips, junctions, and edges; the total hyphal length, area, and volume; or the distribution of branching angles and internodal lengths can be readily extracted from the pixel skeleton or graph representation of the fungal network (116, 144, 173, 175, 199–202). Measures can also be referenced to 2-D space to give tip densities and fractal dimensions (173, 175). Topological network measures, such as node degree, α-index or shortest path metrics, such as betweenness centrality (Fig. 7D), can also be readily calculated and show species-dependent developmental changes over time (144, 173, 199–203).

In more realistic microcosms with patchy resources, in competition with other species, or in the presence of fungivores, there is considerably more variation in spatial network architecture than is captured in a single summary network statistic. Such heterogeneity may be identified by algorithms that identify regions of the network with a higher local density of connections than other regions of the network than would be expected by chance, and are termed "communities" in network parlance. Communities are initially identified by progressively partitioning the network into smaller and smaller units by breaking links depending on the strength of their interaction, by tuning a resolution parameter. There is considerable flexibility in the choice of the interaction term that forms the basis of the analysis, which can be based on measures such as edge volume, conductance, or resilience (204, 205). The profile of key summary statistics, such as the number of communities, or the energy and entropy of the system, as the resolution parameter is changed, are used to define a set of mesoscopic response functions (MRFs) for each network. Networks are then clustered based on an estimate of the distance between the MRFs to give a dendrogram (Fig. 8). This provides a biologically sensible clustering of networks from different species, and also of developmental stages for a particular species, or exposure to different experimental conditions (204, 205). These approaches allow objective groupings of networks across species, treatments, and laboratories. Nevertheless, these methods are in their infancy and the challenge now is to understand whether the groupings can be interpreted from a biological perspective to yield additional insight that cannot be captured from qualitative description of each network alone.

Integrating Structure and Flows Using Modeling

Constructing models that meaningfully represent processes ranging from the micron scale in individual hyphae to hyphal networks operating at an ecologically relevant scale is extremely challenging. At the microscopic scale, sophisticated biophysical models have been developed to explain the growth of individual hyphal tips, including vesicle delivery and viscoelastic wall deformation (42, 206–211), followed by wall aging through cross-linking of wall polymers (4, 212, 213). While, at a macroscopic scale, the growth of fungi in the environment can be modeled by using differential equations to represent changes in the density of fungal mycelia and spore production, along with changing resource density. Such an approach can be effective for modeling the spread of fungal crop pathogens (214), or modeling carbon cycling in the environment (215), but these kinds of model are essentially blind to the fact that fungal biomass grows as an interconnected network. Here, we consider an intermediate scale, namely the growth of individual mycelial networks.

Continuum Models at the Colony Level

One strategy for modeling the interaction between tips, hyphae, growth-limiting nutrients, and inhibitory waste products is to represent the mycelium as a continuum and use differential equations to model processes such as the uptake of nutrients by hyphae, the extension of tips to form hyphae, the formation of new tips through branching, and the loss of tips because of anastomosis. Such models have their roots in the work of Edelstein and coworkers (216), and they have been reasonably successful in explaining the gross morphology of colonies with dense mycelia, which can be observed growing on agar, plant surfaces, or building materials. For example, when *Aspergillus orzae* grows on agar, the morphology of the colony depends on the concentrations of nutrient and agar (217, 218). At low nutrient concentrations, colonies are uniform with a smooth, circular growth front provided that it is difficult for nutrients and waste products to diffuse through the agar. If the agar concentration is decreased, the effective

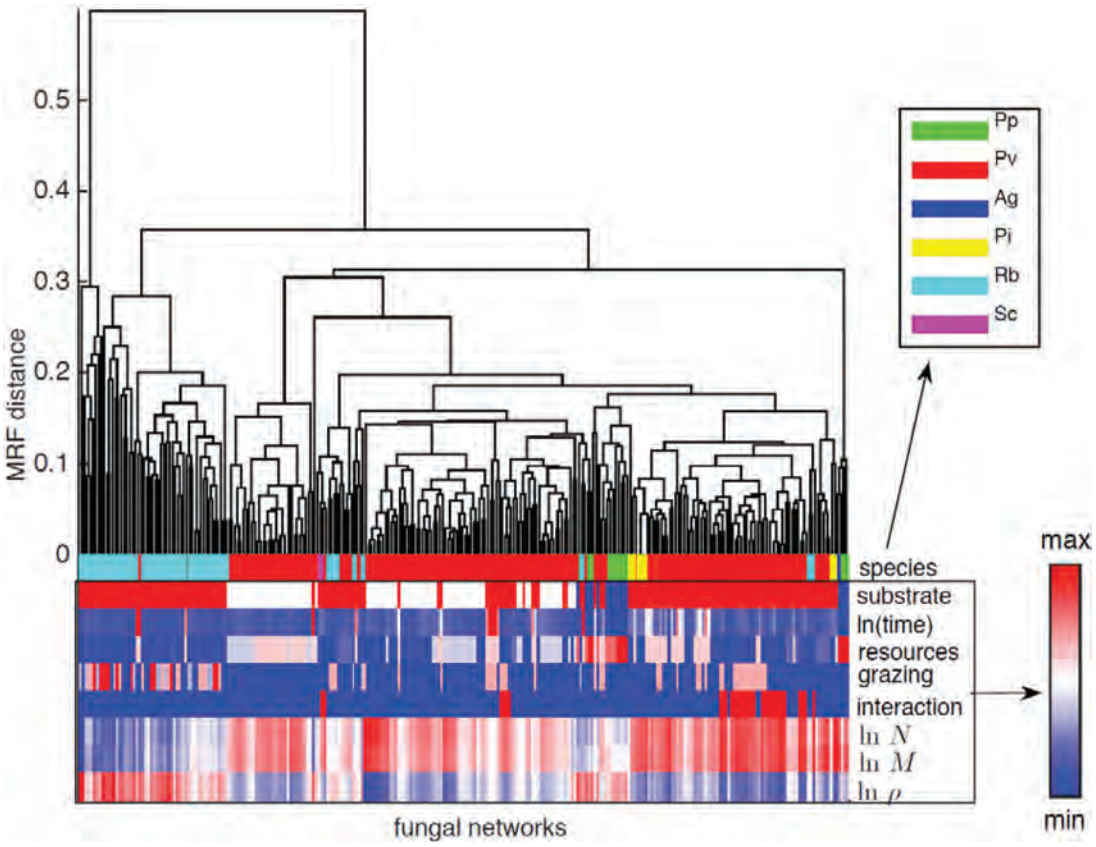


Figure 8 Network taxonomy. Taxonomies of 270 fungal (and slime mold) networks based on community structure using modularity optimization with path score (PS) values as the edge weights (205). The dendrogram was produced from the mesoscopic structure of each network as an indication of how similar different networks are to each other. The species abbreviations are coded as follows: Pp, *Physarum polycephalum*; Pv, *Phanerochaete velutina*; Ag, *Agrocybe gibberosa*; Pi, *Phallus impudicus*; Rb, *Resinicium bicolor*; and Sc, *Strophularia caerulea*. The levels of resources and amount of grazing are color coded from low (blue) to high (red). Substrate is coded as blue for agar, white for sand, and red for compressed, nonsterile soil. Interactions are coded as blue for no interaction, or red grown in competition with *Hypholoma fasciculare*. At the bottom of the figure, the logarithms of number of nodes N, number of edges M, and the edge density $= 2M/N(N-1)$ are also shown. From reference 205 with permission.

diffusion coefficient of waste products and nutrients is increased, and colonies condense into branched forms, with a tortuous, irregular growth front. This morphological instability can be explained by constructing a reaction-diffusion type model that takes into account the build-up of waste inhibitors and the provision of growth-limiting nutrients. Essentially, if the nutrients needed for growth can only be obtained by growing into new territory, a low-density colony will gradually fill the dish, maintaining circular symmetry. If nutrients can diffuse into already colonized regions, this will enable denser growth. However, in that case the build-up of inhibitory molecules can prevent some regions from growing out of the dense core, and any point on the growth front that happens to be further away from the inhibitory core will be more likely to grow, producing positive feedback and an unstable, irregular growth front.

Including Biomass Recycling in Continuum Models

Another feature of fungal physiology that can be incorporated into continuum models is the distinction between mobile and immobile fungal biomass. Metabolically active hyphal tips mature into relatively inert hyphae, or fully vacuolated vessels that are not metabolically active. Uptake of nutrients is generally believed to be greatly reduced behind the hyphal tips, and, in some

species, hyphae develop into cords or metabolically inert transport vessels that are highly insulated from the environment (17, 35, 219). Although some biomass is static, the cytoplasmic contents of the fungal colony is free to move by pressure-driven mass flows, diffusion, or various mechanisms of active transport. Furthermore, some internal resources may be remobilized, as parts of the fungal colony are broken down and recycled to fuel further growth (see "Biomass recycling" above). Energetic arguments suggest that the process of recycling is of much greater benefit to organisms that live on recalcitrant or nutrient-poor substrates (31), and modeling suggests that the rate of recycling may also have a significant impact on colony morphology (142). There are three extreme morphologies: (i) "fairy rings," with an annulus of high-density growth surrounding a region with low or zero density; (ii) colonies of relatively constant density; or (iii) rings that alternate between higher and lower densities (220; see "Colony dynamics and behavior" above). The distribution of densities of fungal growth depends on both the nutrient environment and the species in question, and models indicate the importance of nutrient transport and biomass recycling in determining such morphologies (24, 26, 142). The fact that fungal growth is space filling is critically important, because the capacity to take up nutrients, water, or pollutants from the environment is proportional to the surface area. There is no reason to suppose that the total surface area of a fungal colony is a simple function of its diameter, which suggests that fungal growth and its environmental consequences may not be well represented by assuming some density of growth. One approach is to represent the space-filling capacity of fungi in terms of fractal dimension (188, 221–224) as the dimension of a space will influence the dynamics of any exchange process (225) (see "Fractal measures and hyphal coverage" above).

Spatially Explicit Network Models

A promising but computationally intensive approach is to adopt a spatially explicit representation of all the hyphae in a network (26, 29, 226). Each hyphal tip can be associated with a location and a direction, and the movement of those tips can be modeled so that tip velocity and changes in tip direction recapitulate empirical observations. These agent-based models can either be constrained to a lattice, or, at the expense of additional computational cost, the orientation of hyphal tips can be lattice free (28, 30). In all such "tip-and-trail" models, hyphae are present wherever the moving tips have passed, and those locations can be given traits such as internal and external concentration of growth-limiting resource. This approach enables the modeling of hyphal fusion, which is assumed to occur whenever a tip grows into a location that is already occupied by hyphae or tips.

Branching angle can also be modeled to recapitulate empirical observation, but there are fundamental difficulties in modeling branching frequency. One approach is to simply impose the distribution between branch points that has been observed experimentally (226). This may be appropriate when branching rate is constant and all branching events are apical or subapical because in that case it is relatively easy to measure branching frequency and the location of branching events is relatively well determined. Such a model can be used to determine the relationship between branching rate, branching angle, and the expected distribution of fungal density for colonies of various sizes (226), but in cases where the environment is heterogeneous and branching rate is not constant, it is not clear which branching rate should be imposed. An alternate approach is to suppose that tip growth and branching rate are a function of the internal concentration of some growth-limiting resource (29, 227). Such models effectively reveal the importance of nutrient transport, but the internal concentration of nutrients will depend on the scale and mechanisms of nutrient uptake and translocation about which we still have little quantitative information (16, 56, 147, 228). Thus, it is not clear whether these models represent the mechanisms of nutrient transport with sufficient accuracy, nor is it clear whether they capture the actual relationship between branching rates and local conditions. Crucially, the connected nature of mycelial networks means that local behavior can be affected by conditions in remote parts of the colony, especially by the translocation of nutrients (32, 219, 229, 230).

Furthermore, because the flow of cytoplasmic contents is relatively unimpeded, turgor pressure will tend to equalize rapidly across the colony. Hence, turgor pressure and the rate of water uptake necessarily reflect the osmotic potential of the entire colony and its environment, not just the local conditions (49, 231). This implies that the behavior of the colony is dependent on the interaction between the genotype and a complex spatial convolution of environmental conditions (26, 142, 232, 233). It should also be noted that not all cords are equally important when a dynamic process occurs on a network. In a heterogeneous environment some cords will carry more resources than other, nearby edges (28), and empirical observations confirm that the distribution of velocities of fluid flow within a fungal network are highly heterogeneous, with some cords

carrying much larger mass flows than others (32, 33, 67). This heterogeneity is presumably related to the fact that some hyphae branch and form hyphal aggregates or cords, while other hyphae are degraded and recycled. There is some evidence that cords which carry greater currents are more likely to thicken (33), but there is a substantial body of open questions concerning the developmental logic of fungal networks.

Comparison with Other Types of Transportation Networks

Given the importance of nutrient transport, it is worth considering how fungal networks compare with other transportation networks (32, 144, 219). In particular, modeling may be able to shed some light on the trade-offs involved in forming cross-links, because it is notable that some fungal networks contain numerous loops, while other fungi essentially grow as branching trees (16, 199, 201). There is a considerable literature on the optimal design of distribution networks, and fractal branching trees are known to be optimal for the case where a single source is connected to multiple sinks (234–236). Furthermore, when a metabolism-limiting resource is distributed through a fractal branching tree, we should expect to see scaling effects such that larger organisms have a lower metabolic rate. As theory predicts, there is evidence in the animal kingdom that metabolic rate is proportional to $M^{3/4}$, where M represents the mass of the animal (234, 235). However, while mammals use a cardiovascular system to distribute oxygen, glucose, and other metabolism-limiting substances throughout the body from a single heart, fungal colonies generally cannot be represented as a single-source system. Nutrients may be gathered from across the colony, and such organisms may thrive by foraging phosphate from one location and nitrogen from another, and distributing both resources throughout the mycelium (147, 219). Hence, in the case of fungi, the distance that determines the scale of the transport challenge is not the radius or the mass of the colony, but the typical distance between the site of nutrient uptake and the place where those nutrients are consumed.

Whatever the spatial distribution of sources and sinks, any efficient flow pattern must be such that, at every point, the flow moves materials away from the source and toward the sink. In other words, although there may be loops in the network, optimally efficient flows always transport materials in a directed manner. This feature of efficient material flows is common to many definitions of "efficient," and it is inevitable when the flows are driven by differences in potential. That is because, at every point in any given network, material moves from regions of high potential to low potential. Hence, material can never backtrack, because that would require movement against the potential gradient. However, although it is most efficient for particles to move in a directed manner, there are circumstances in which the most efficient network is one that contains loops. In particular, if a network has to function in the face of random damage, or a network needs to connect a fluctuating distribution of sources and sinks, the most efficient choice of the network may be one that contains loops (237). Further modeling may help to elucidate the trade-offs that arise between the conflicting demands of efficient directed transport and efficient mixing. In many cases, the primary interest is in the functional consequences of fungal growth, such as the production rate of desirable compounds, the rate of nutrient or pollutant uptake, rates of soil acidification, wood degradation, and so on (227). Some of these macroscopic rates of interest have been connected to microscopic fungal parameters, such as branching rate or fungal density, but more effective predictive models may require a deeper understanding of how fungal development shapes and responds to the environment in which growth occurs. The increasingly quantitative nature of experimental data concerning tip growth, branching, nutrient transport, and cytoplasmic flow should provide valuable material for future modeling efforts (116), and advances in imaging technology will surely enable a more detailed analysis of the architecture and functional significance of developing fungal networks.

Network Robustness and Resilience

In natural environments mycelial networks are subject to physical damage and attack by fungivores. The intrinsic robustness of the network at a particular time point is determined by the network architecture, including thickness and toughness of hyphae and mycelial cords, palatability to grazers, and extent of interconnectedness. In particular, networks with loops formed by anastomosis are more robust, because they already maintain alternative routes for nutrient translocation to circumvent damaged regions. Fungi can also respond to damage or attack by network remodeling leading to greater resilience. Experiments can be conducted with real networks in the presence of fungivores, or by using an *in silico* approach based on network analysis in other domains, whereby a property of the network, such as the largest connected component or the predicted transport efficiency, is calculated as cords are removed according to some rule. Thus, long thin cords might be removed first to simulate grazing by collembola with

small mouth parts, or a local region of the network might be deleted as a chunk to simulate grazing by wood lice.

Experiments in Soil Microcosms

The effects of invertebrates grazing on mycelia of cord-forming wood decay basidiomycetes have been studied extensively in microcosms (24 × 24 cm or 50 × 50 cm) of compressed nonsterile woodland soil (238–240). Intense grazing can completely destroy mycelia and prevent them from growing out of organic resources, but less intense grazing can dramatically alter mycelial morphology and physiology, in different ways depending on the grazing species, the fungus, and the grazing intensity. Mycelia are grazed in different regions and to different extents depending on fungal species: collembola grazing on the relatively unpalatable *H. fasciculare* tends to be at hyphal tips; in contrast, *R. bicolor* extending from beech wood is indiscriminately grazed, and, with *P. velutina*, fine hyphae within the colony and hyphal tips are grazed (241). While microfauna and mesofauna often graze on fine hyphae, macrofauna such as woodlice (*Oniscus asellus*) graze in large swathes including severing thick cords (242).

Susceptibility to grazing also depends on the age and stage of development of the mycelium and the resources to which it has access. Mycelia comprising individual hyphae or thin cords, at early stages of development or supported by a dearth of resources, are more prone to grazing than mycelia that have formed highly sclerotized cords interconnecting between woody resources (170, 239). Mycelial systems occupying large (50 × 50 cm) trays of soil had similar hyphal coverage after several months irrespective of whether or not they had been grazed by collembola (170), apparently because collembola grazed on the fine hyphae that would have died anyway as the system became more open. Greater mycelial interconnectedness, i.e., more cross-links forming tangential connections, also limits the effects of grazing and other damage (Fig. 5 and 9) (169, 243). Connectedness is greater in some species than others, e.g., greater in *Phallus impudicus* than *P. velutina* (see above), in mycelia developing from larger resources and in more mature systems (144, 169, 243, 244).

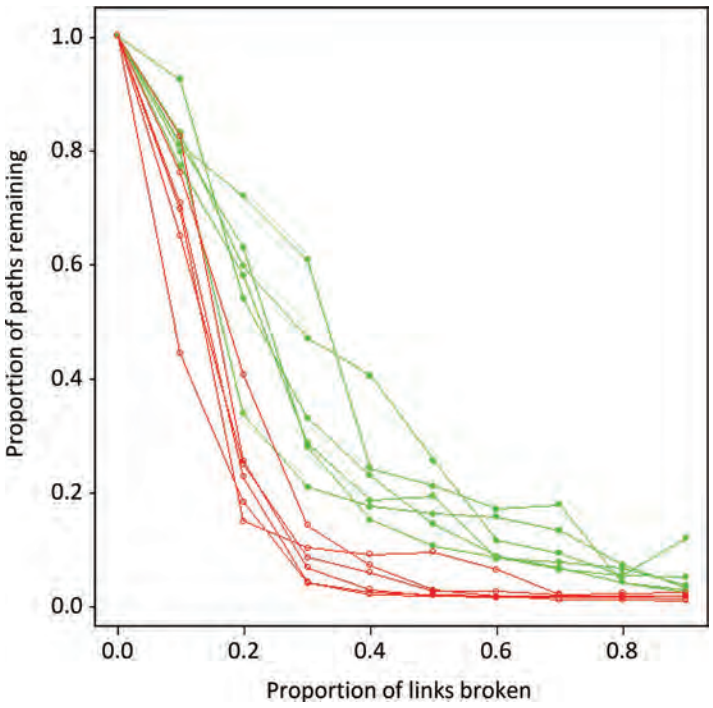


Figure 9 *In silico* evaluation of network robustness. Networks of *Phanerochaete velutina* (red) and *Phallus impudicus* (green) were attacked *in silico* by progressively removing links in the network at random, and calculating the number of paths in the network that still remain. In each case, five examples are shown. Note that the *P. velutina* network breaks down more rapidly than the *P. impudicus*. From D. A'Bear, L. Boddy, and M. D. Fricker, unpublished data.

Effects of grazing depend not only on the intrinsic robustness but also on how mycelia respond to damage or grazing, i.e., resilience. Although extension rate often decreases as a result of invertebrate grazing (239), it sometimes increases, switching from slow, exploitative to fast, explorative growth (242). These changes sometimes occur at or close to the site of grazing, further away, and in all directions around a mycelial margin. The latter is invertebrate density dependent (239) and has been interpreted as compensatory growth, similar to that which occurs in plants during herbivory (238). During interactions between mycelia of *P. velutina* and *H. fasciculare* growing across soil, grazing by collembola curtailed growth of the former except in the region of the opponent, where rate of overgrowth rapidly increased (243). Remarkably, although collembola grazing of *P. velutina* tends to reduce interstitial hyphal biomass overall, some of these fine hyphae survive, and thicken, increasing the local level of cross-connectivity, and hence increasing robustness (169).

Network Risks: Control of Systemic Infection

Network organization may be useful for long-distance transport and coordinated colony behavior, but also poses a risk of rapid systemic infection. Indeed, approximately 30 to 80% of fungi are thought to harbor persistent systemic mycovirus infections that spread horizontally via hyphal anastomosis between compatible fungal hosts, or vertically through spore formation, with no extracellular mode of infection (245, 246).

FUTURE PROSPECTS

Extension of Network Analysis to 3-D

It is already possible to collect sets of tiled 3-D confocal images of fungi growing on agar with sufficient resolution to identify individual hyphae, and sufficient spatial and temporal scales to map colony development over a period of days, which gives a much richer source of information for modeling (116). Recent progress has also been made on 3-D imaging of fungi in wood or soil with sub-micron resolution using X-ray computed tomography (247–250). However, manual or automated segmentation and analysis routines are still challenging, as the invasive hyphae can be very narrow and it is currently difficult to achieve sufficient contrast to segment fungal hyphae from the woody tissue (247, 248, 250). Imaging mycelial networks in soil has used osmium vapor staining and use of X-ray translucent polystyrene substrate to create sufficient contrast (249). Nevertheless, at this point, it is not possible to extract a connected 3-D network from either substrate, but it is anticipated that further methodological improvements will overcome some of the current technological challenges.

Intercellular and intracellular hyphal networks formed during host-mycorrhizal interactions have been successfully imaged at cellular resolution using confocal microscopy after labeling with various fluorophores (133, 251–253), using label-free imaging (254), by *in situ* hybridization (255), or following combined KOH maceration, resin embedding, physical sectioning, and confocal imaging of fluorescently labeled wheat germ agglutinin lectin (256, 257). Quantitative measurements of volumes and surface areas are possible, even from dense arbuscules (251). Techniques to visualize arbuscular mycorrhizal networks by sandwiching between Millipore filters give impressive images of the mycelial network and have allowed quantitation of hyphal lengths and number of anastomoses (258, 259), and could easily be subject to network analysis. Likewise, cord-forming ectomycorrhizal networks can be imaged and analyzed by using the same approaches developed for saprotrophic systems (Fig. 10), although it is more complex to segment the fungal mycelium from the root because they span different scales and the color discrimination from each other and the soil is difficult.

In a similar manner, pathogens invading their host can be labeled by using nonspecific stains or fluorochromes or by following expression of fluorescent proteins (260–262). Lesion development can be followed by automated segmentation and analysis by using the HyphArea program (191), which has the potential to provide a complete, quantitative representation of the invasive hyphal network. The length scale that most pathogen infections operate over is quite limited (millimeters to centimeters), which suggests that growth and internal nutrient movement can be accomplished easily by diffusion and cytoplasmic streaming rather than by mass flow.

Network Analysis and Fitness Traits

The network formed by different species is recognizable at a macroscopic level, and tools to quantify network architecture and dynamics are emerging. At this point, we do not know which metrics may best describe network organization and how these relate to fitness (263), but it is not unreasonable to propose that each species operates a slightly different set of local rules to balance growth, transport efficiency, recycling, resilience, and reproduction that collectively maximize the long-term global success of the organism (31, 264). Thus, network

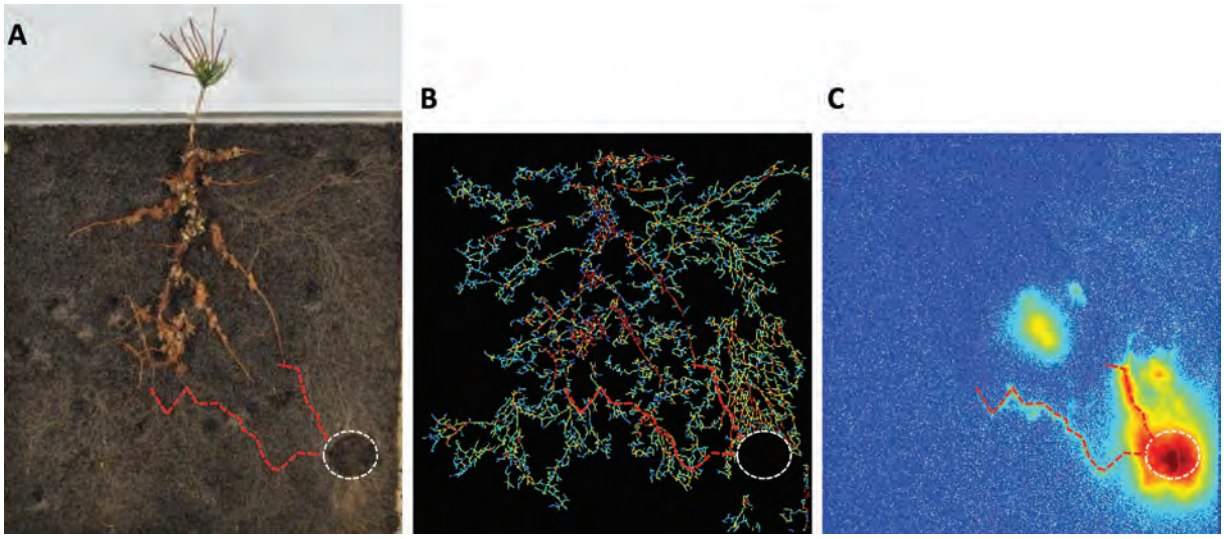


Figure 10 Network analysis of the ectomycorrhizal fungus *Paxillus* on pine. (**A**)*Pinus* seedling infected with *Paxillus*. The dotted white circle marks the loading site for radioactive ^{14}C-AIB. The dotted red lines indicate the routes for preferential transport of isotope to the roots from C. (**B**) Automated extraction of both the plant root and extra-radical mycorrhizal network using phase-congruency enhancement, watershed segmentation, and cord width measurement. (**C**) Scintillation image of nutrient movement along selected pathways from the fungus to the plant root. From R. Tajuddin, D. Johnson, and M.D. Fricker, unpublished data.

analysis may help to provide quantitative measures of traits that fulfill the criteria of ecological versatility, wide scope throughout the fungal kingdom, and measurability set out by Aguilar-Trigeros et al. (39).

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Fungal Ecology

III

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The Geomycology of Elemental Cycling and Transformations in the Environment

16

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INTRODUCTION

The significance of fungi in natural environments is extensive and profound. Their most obvious roles are as decomposers of organic materials and as animal and plant pathogens and symbionts. It is therefore obvious that they are of major importance in the global carbon cycle through such activities and as important determinants of plant growth and productivity. However, their importance in terms of nutrient and element cycling greatly extends beyond these core activities, and they are involved in the biogeochemical cycling of many other elements and substances, as well as many other related processes of environmental significance. The growing discipline of geomicrobiology addresses the roles of microorganisms in geological and geochemical processes (1, 2), and geomycology can be considered to be a part of this topic that focuses on the fungi (3, 4). The often clear demarcation between mycological and bacteriological research has ensured that the geoactive properties and significance of fungi have been unappreciated in wider geomicrobiological contexts. The range of prokaryotic metabolic diversity found in archaea and bacteria, including their abilities to use a variety of different terminal electron acceptors in respiration and effect redox transformations of many metal species (5, 6), has also contributed to a narrow overall view of the significance of eukaryotic organisms in important biosphere processes. A recent collection of geomicrobiology review articles managed to completely exclude fungi (as well as algae), even to the extent of defining "microbes" as being only bacteria and archaea (7). Nevertheless, appreciation of fungi as agents of geochemical change is growing, and their significance is being discovered even in locations not usually regarded as prime fungal habitats, e.g., rocks, acid mine drainage, deep aquatic sediments, hydrothermal vents, and the igneous oceanic crust (8–11). Their significance as bioweathering agents of rocks and minerals is probably better understood than bacterial roles (12), and this ability is of prime importance in the weathering of human structures in the built environment and cultural heritage (13–15). On the positive side, the geoactive properties of fungi can be used for human benefit, and several aspects may contribute to providing solutions to several important global challenges. Geomycology is relevant to reclamation and revegetation of polluted habitats, bioremediation, nuclear decommissioning and radionuclide containment, biorecovery of important elements, and the production of novel biomaterials. This chapter outlines important geoactive properties of fungi in relation to important environmental processes, their positive and negative applications, and their impact on human society.

THE FUNGAL HABITAT

Fungi are ubiquitous components of the microbial communities of any terrestrial environment, including such hostile habitats as the Arctic, hot deserts, and metal-rich and hypersaline soils (16). They are significant inhabitants of the aquatic environment as decomposers of organic matter but are also involved in other elemental cycles, e.g., manganese oxidation (17). Fungi are ubiquitous in habitats polluted by xenobiotics, toxic

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metals, and radionuclides, both terrestrial and aquatic, as well as leachates and other solid and liquid wastes (18). In such habitats, fungi may exhibit a variety of mechanisms that determine tolerance and survival and which are also components of elemental cycles for pollutant elements (18). These "extreme" locations may also act as a reservoir of novel organisms with unusual properties. For example, acid mine drainage is now known to harbor fungal communities, the preponderance of earlier research on this habitat being devoted to prokaryotes, and some isolates possess unusual element bioaccumulation properties. New species include *Fodinomyces uranophilus* and *Coniochaeta fodinicola* from uranium mine locations that can bind mobile uranium (10, 19) and a *Penidiella* sp. from an acidic abandoned mine location that was capable of accumulating rare earth elements such as dysprosium (Dy) (20). A global fungal role in biogeochemical cycling of the elements (e.g., C, H, N, O, P, S, metals, metalloids) is therefore obvious and is interlinked with the ability to adopt a variety of growth, metabolic and morphological strategies, adaptive capabilities to environmental extremes, and symbiotic associations with animals, plants, algae, and cyanobacteria (16, 21, 22).

The ability of many fungi to grow oligotrophically by scavenging nutrients from the air and rainwater helps them survive on stone and rock surfaces which are considered to be an inhospitable environment (9, 23). In addition, organic and inorganic residues on mineral surfaces or within cracks and fissures can act as nutrient sources in the subaerial rock environment (24). One of the most successful means for fungi to survive in the extreme subaerial environment is underpinned by their symbiotic associations with algae and cyanobacteria as lichens, where the phototrophs provide a source of carbon and protection from light and irradiation (24, 25). Lichens enable colonization of a wide range of environments including those at climatic extremes such as the Arctic and Antarctic, exposed rock surfaces, and dry deserts.

In the deep subsurface, the research emphasis is mostly on prokaryotes, but the presence of fungi is now well known (11, 26–28). Fungi occur in abundance and high diversity in such varied environments as deep-sea sediments (29), hydrothermal vents (30, 31), and methane cold-seeps (29, 32). They are now also known as abundant inhabitants of the igneous oceanic crust, which has consequently been described as the largest fungal habitat on Earth (11). Fungi seem to play an important ecological role in the igneous oceanic crust, because they exist in symbiosis with chemolithotrophic prokaryotes, decompose organic matter, dissolve and form minerals, and therefore are involved in the cycling of elements (11, 33, 34). Fossilized microorganisms have been observed in drilled cores and dredged samples from the ocean floor, with a majority of these findings representing fungi (34, 35). These fungi existed in a close symbiotic-like relationship with two types of prokaryotes, which appeared to use the structural framework of the mycelium for their growth (34). It therefore seems clear that geomycological processes are significant in a wide range of biosphere habitats, including those traditionally thought to be inimical to fungal growth and development (36).

ORGANIC MATTER DECOMPOSITION AND BIOGEOCHEMICAL CYCLING OF COMPONENT ELEMENTS

Organic matter decomposition is the attribute most commonly associated with fungi and is a major contribution to global biogeochemistry as well as the spoilage of foodstuffs and organic materials (21, 36). Fungal processes represent a potential control point in the global carbon cycle (37). To some extent, research on this aspect of chemoorganotrophic metabolism has obscured the wider global implications of decomposition in terms of the cycling of other elements and nutrients, and also contributed to a lack of attention to fungal roles in wider geochemical cycles.

Most biogeochemical attention on fungi has been given to carbon and nitrogen cycles, and the ability of fungi to utilize a wide spectrum of organic compounds is well known. Simple compounds such as sugars, organic acids, and amino acids can easily be transported into the cell, while more complex molecules are first broken down into smaller molecules by extracellular enzymes before cellular entry. Such compounds include natural substances such as cellulose, pectin, lignin, lignocellulose, chitin, and starch and anthropogenic products such as hydrocarbons, pesticides, and other xenobiotics (21, 37). Organometals (compounds with at least one metal-carbon bond) can also be attacked by fungi. Degradation of organometallic compounds can be carried out by fungi either by direct enzymic action or by facilitating abiotic degradation, e.g., by alteration of external pH through metabolite excretion. Tributyltin oxide and tributyltin naphthenate may be degraded to mono- and dibutyltins by fungal action, inorganic Sn^{II} being the final degradation product (38). Organomercury compounds may be detoxified by conversion to Hg^{II} by fungal organomercury lyase, the Hg^{II} being subsequently reduced to Hg^{0} by mercuric reductase, a system analogous to that found in mercury-resistant bacteria.

Some fungi have remarkable degradative properties, and ligninolytic fungi, such as the white rot *Phanerochaete chrysosporium*, can degrade xenobiotics including aromatic hydrocarbons, chlorinated organics, polychlorinated biphenyls, nitrogen-containing aromatics, and many other pesticides, dyes, and xenobiotics (39, 40). Such activities are important in polluted habitats and have been applied in bioremediation where ligninolytic fungi have been used to treat soil contaminated with substances such as pentachlorophenol (PCP) and polynuclear aromatic hydrocarbons (PAHs) (21, 41–43). In many cases, xenobiotic-transforming fungi need additional utilizable carbon sources for cometabolism because although capable of degradation, they cannot adequately utilize these substrates as an energy source. Inexpensive utilizable lignicellulosic wastes such as corn cobs, straw, and sawdust can therefore be used as nutrient additions for enhanced pollutant degradation. Wood-rotting and other fungi have also received considerable attention forthe bleaching of dyes and industrial effluents, and biotreatment of various agricultural wastes such as forestry, pulp, and paper by-products; sugar cane bagasse; coffee pulp; sugar beet pulp; apple and tomato pulp; and cyanide (42).

Fungi are highly important in the degradation of naturally occurring complex molecules in terrestrial and aquatic habitats. Since around 95% of plant tissue is composed of carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulfur, decomposition activities of fungi are clearly important in relation to redistribution of these elements between organisms and environmental compartments. As well as C, H, O, N, P, and S, another 15 elements are typically found in living plant tissues: K, Ca, Mg, B, Cl, Fe, Mn, Zn, Cu, Mo, Ni, Co, Se, Na, and Si. However, all 90 or so naturally occurring elements may be found in plants, mostly at low concentrations, although this may be highly dependent on environmental conditions. These include toxic and inessential metals and metalloids including As, Hg, Pb, and U. Some plants accumulate relatively high concentrations of metals such as Ni and Cd. Plant metal concentrations may reflect environmental conditions and provide a bioindicator of toxic metal pollution or a metalliferous substrate. Metal-accumulating plants are also receiving attention in bioremediation (i.e., phytoremediation).

Similar concepts of element cycling also relate to animal and microbial biomass. Animals also contain multiple elements in varying amounts. The human body (like other organisms) is mostly water, and around 99% of the mass comprises oxygen, carbon, hydrogen, nitrogen, calcium, and phosphorus. However, many other elements are present in lower amounts including substances taken up as contaminants in food and water. It follows that decomposition and degradative and pathogenic activities of fungi are linked to the redistribution and cycling of all these constituent elements on local and global scales. There must be a fungal component, therefore, in the biogeochemical cycles of virtually all biomass-associated elements (3, 21). The release of elements and nutrient moieties through degradation makes them available to other microorganisms and plants, as does chemical interaction with the environmental pool of different chemical species.

FUNGAL INTERACTIONS WITH THE INORGANIC ENVIRONMENT: ROCKS, MINERALS, AND METALS

Bioweathering

Rocks and minerals represent a vast reservoir of elements, many essential for life, and such elements must be released in forms that may be assimilated by the biota. These include essential metals as well as elements such as S and P (3, 44). Many important microbial processes are influenced by minerals including nutrient acquisition, cell adhesion, and biofilm formation (45). Essential nutrients and environmental contaminants sorbed to mineral surfaces can be acquired or removed by organisms including metals and organic compounds (46, 47). Other elements and nutrients may be released from minerals as a result of bioweathering, and fungi have notable activities in this context (3, 16, 24, 48).

Bioweathering can be defined as the erosion, decay, and decomposition of rocks and minerals mediated by living organisms. Fungi are well suited as geoactive weathering agents since they possess a variety of growth, metabolic, and morphological strategies and can be resistant to a range of environmental extremes such as metal toxicity, UV radiation, and desiccation. Their mutualistic associations with plants (mycorrhizas), algae, and cyanobacteria (lichens) are particularly significant geoactive agents (3, 16, 24, 48). The ability of fungi to translocate water, ions, and nutrients within the mycelial network is another important feature for exploiting heterogeneous environments (49–51).

Fungi appear to be ubiquitous components of the microbiota of all rocks, building stone, and concrete, and have been reported from a wide range of rock types, e.g., limestone, marble, granite, sandstone, basalt, gneiss, dolerite, and quartz, even from the most extreme environments (9, 16, 48). Rock surfaces may

be subject to moisture deficit and nutrient limitation, although many species can tolerate extremes of UV irradiation, salinity, pH, and water potential (16, 24, 25, 48, 52). Nutrients can be scavenged from the atmosphere and rainwater, and they also use organic and inorganic residues on surfaces or within cracks and fissures, waste products of other microorganisms, decaying plants and insects, dust particles, aerosols, and animal feces as nutrient sources (24). Fungi may receive protection from environmental extremes by the presence of melanin pigments and mycosporines in their cell walls and by the production of mucilaginous exopolymeric substances that may entrap inorganic particulates, e.g., clay minerals, providing further protection (9, 53). Fungal interactions with rock-mineral substrates can result in dissolution and biodeterioration but also the formation of patinas, films, varnishes, and crusts (3, 9). In soil, fungus-mineral interactions are also an integral component of environmental cycling of elements and nutrients (4, 21).

Biomechanical deterioration of rocks and minerals can occur through hyphal penetration and burrowing into decaying material and along crystal planes in, e.g., calcitic and dolomitic rocks (3, 24, 54). Intracellular turgor pressure may be a significant factor in biomechanical disruption (55, 56). Spatial exploration of the environment to locate and exploit new substrates is facilitated by a range of sensory responses that determine the direction of hyphal growth such as thigmotropism (or contact guidance) (57). Biochemical weathering of rocks and minerals can occur through excretion of geoactive metabolites (58, 59), and this is believed to be a more significant process than mechanical degradation, although a combination of mechanisms is often likely. This can result in pitting and etching of surfaces to complete dissolution of mineral grains (60–62). Bioweathering is a highly significant process and has direct consequences not only for rock and mineral dissolution, but for the mobilization and immobilization of metals, nutrient release, and the formation of secondary minerals (2, 3).

Metal Mobilization

Metal mobilization from rocks, minerals, soil, and other substrates can be a consequence of protonolysis, carbonic acid formation from respiratory CO_2, complexation by Fe^{III}-binding siderophores and other excreted metabolites (e.g., amino acids, phenolic compounds, and organic acids), and methylation (e.g., Hg, Se, and As), which can result in volatilization. Fungus-excreted carboxylic acids can attack mineral surfaces, providing protons as well as a metal-chelating anion, e.g., citrate (58). Oxalic acid can leach metals that form soluble oxalate complexes, e.g., Al and Fe (63). Solubilization mechanisms can result in metal mobilization from toxic metal-containing minerals, e.g., pyromorphite [$Pb_5(PO_4)_3Cl$], contaminated soil, and other solid wastes (64–66). Fungi may also mobilize metals and attack mineral surfaces by redox transformations: Fe^{III} and Mn^{IV} solubility is increased by reduction to Fe^{II} and Mn^{II}, respectively. Fungal reduction of Hg^{II} to volatile elemental Hg^0 has also been recorded (67). As discussed earlier, metals may be mobilized from organic substrates during decomposition (21).

Metal Immobilization

Fungi are effective accumulators of metals and related substances. Important mechanisms include biosorption to cell walls, pigments, and exopolymers, intracellular transport, accumulation and sequestration, and bioprecipitation on and/or around hyphae (3, 53, 68–76). Living and dead fungal biomass are effective biosorbents for a variety of metals including Ni, Zn, Ag, Cu, Cd, and Pb as well as actinides, e.g., U and Th, with a variety of functional groups being involved (24, 53, 76). The presence of chitin, and pigments such as melanin, may enhance the ability of fungal biomass to act as a biosorbent. Fungal biomineralization processes lead to metal immobilization as biominerals or elemental forms, as described below (3).

Biomineralization

Biomineralization is the processes by which organisms form minerals. Biologically induced mineralization is where the organism modifies the local microenvironment, creating conditions amenable for extracellular chemical precipitation of mineral phases. The organism does not appear to control the biomineralization process in biologically induced mineralization, while a great degree of control over biomineralization is exerted in biologically controlled biomineralization, e.g., the complex cellular biomineral structures found in certain other eukaryotes such as diatoms (77). Fungal biomineralization therefore usually refers to biologically induced mineralization. This can result from the bioweathering mechanisms discussed previously such as redox transformations and metabolite excretion (78, 79) and organic matter decomposition where released substances reprecipitate with metals in the microenvironment and *vice versa* (4, 21, 80–82). Fungal surfaces provide many reactive sites for sorption (i.e., biosorption), and this can also lead to the formation of mineral precipitates (2, 71, 83).

Common Mineral and Biomineral Transformations by Fungi

Fungi are involved in many environmental mineral transformations at differing scales (84–86). They are clearly a very important group of geoactive organisms, especially when considering their ubiquity and capacity for production of mineral-transforming metabolites, their symbiotic associations, and the aforementioned consequences of their major significance in organic matter decomposition (4, 5).

Carbonates

Insoluble carbonates may be broken down by fungal attack, usually the result of acid formation (87–89). Such activity is particularly evident on limestones and marble used in building construction but can also occur in natural limestone (88, 90). Fungal attack on carbonates (dolomites and limestones) can result in transformation of these substrates to dolomite [$CaMg(CO_3)_2$], glushinskite ($MgC_2O_4.2H_2O$), weddellite ($CaC_2O_4.2H_2O$), whewellite ($CaC_2O_4.H_2O$), and possibly struvite ($NH_4MgPO_4{\cdot}6H_2O$) (91).

Certain fungi can deposit calcium carbonate extracellularly (92–95). Calcite ($CaCO_3$) and calcium oxalate monohydrate (whewellite; $CaC_2O_4.H_2O$) were precipitated on hyphae of *Serpula himantioides* when grown in simulated limestone microcosms (93). Urease-positive fungi degrade urea-liberating carbonate (96). This process results in the precipitation of metal-containing carbonates, which provides a means of metal immobilization and biorecovery (94). Incubation of *Neurospora crassa* in urea-containing media resulted in the formation of calcite, as well as carbonates containing other metals. When a carbonate-laden *N. crassa* culture supernatant was mixed with $CdCl_2$, the Cd was precipitated in the form of highly pure otavite ($CdCO_3$) (94). After incubation in media containing urea and $CaCl_2$ and/or $SrCl_2$, *Pestalotiopsis* spp. and *Xepiculopsis graminea* (syn. *Myrothecium gramineum*), isolated from calcareous soil, precipitated calcite ($CaCO_3$), strontianite ($SrCO_3$), vaterite in different forms [$CaCO_3$, $(Ca_xSr_{1-x})CO_3$], and olekminskite [$Sr(Sr,Ca)(CO_3)_2$], suggesting that urease-positive fungi could play an important role in the environmental fate, bioremediation, or biorecovery of Sr or other metals and radionuclides that form insoluble carbonates (95). *Paecilomyces javanicus* mediated the formation of an unknown lead mineral phase after incubation in liquid media with lead shot. After 2 weeks of incubation, precipitated mineral phase particles were found to contain plumbonacrite [$Pb_{10}(CO_3)_6O(OH)_6$]. However, after 4 weeks of incubation, the lead particles that accumulated inside the fungal pellets were transformed into a white mineral phase composed of lead oxalate (PbC_2O_4), hydrocerussite [$Pb_3(CO_3)_2(OH)_2$], and a new lead hydroxycarbonate mineral species, thus revealing novel steps in lead carbonation by fungi (97).

Oxalates

Calcium oxalate is the most common form of oxalate in the environment, occurring as the dihydrate ($CaC_2O_4.2H_2O$, weddellite) or the more stable monohydrate ($CaC_2O_4.H_2O$, whewellite) (59, 79). Calcium oxalate can be associated with free-living, pathogenic, and plant symbiotic fungi and lichens and is formed by precipitation of soluble calcium as the oxalate (59, 61, 87, 98, 99). Fungal calcium oxalate can exhibit a variety of crystalline forms (tetragonal, bipyramidal, plate-like, rhombohedral, or needles) (100). Calcium oxalate has an important influence on soil biogeochemistry, acting as a calcium reservoir, and can also influence phosphate availability. Fungi can produce many other metal oxalates on interacting with a variety of metals and metal-bearing minerals, e.g., Ca, Cd, Co, Cu, Mg, Mn, Sr, Zn, Ni, and Pb (3, 59, 64, 79, 101–103). The formation of toxic metal oxalates may contribute to fungal metal tolerance (53, 102). In many arid and semiarid regions, calcareous soils and near-surface limestones (calcretes) are secondarily cemented with calcite ($CaCO_3$) and whewellite (calcium oxalate monohydrate, $CaC_2O_4.H_2O$) and the presence of fungal filaments biomineralized with these substances has been reported (52). Calcium oxalate can also be degraded to calcium carbonate, and this may again cement preexisting limestones (104). Other experimental work has demonstrated fungal precipitation of secondary calcite, whewellite, and glushkinskite ($MgC_2O_4.2H_2O$) (3, 16, 48, 93). Fungal attack on a dolomitic and seawater substrate resulted in the formation of Ca-oxalates (weddellite, $CaC_2O_4.2H_2O$; whewellite, $CaC_2O_4.H_2O$) and glushinskite ($MgC_2O_4.2H_2O$) (105).

Oxides

Several fungi can oxidize Mn^{II} to $Mn^{IV}O_2$, including *Acremonium* spp. (17, 106, 107). Fungal oxidation is probably nonenzymatic in many cases, although involvement of laccase and/or multicopper oxidases has been shown in ascomycetes (17, 106). Non-enzymatic microbial Mn^{2+} oxidation may be effected through production of organic acids such as citrate, lactate, malate, gluconate, or tartrate. Some fungi can oxidize Mn^{II} and Fe^{II} in metal-bearing minerals such as siderite ($FeCO_3$) and rhodochrosite ($MnCO_3$), resulting in their precipitation as oxides (108). Manganese and iron oxides

are major components (20 to 30%) along with clay (~60%) and various trace elements in desert varnish (9, 108). Oxidation of Fe^{II} and Mn^{II} by fungi can lead to the formation of dark patinas on glass surfaces (109). Manganese-reducing microbes may mobilize oxidized manganese, releasing it into the aqueous phase. Most of those fungi that reduce Mn^{IV} oxides reduce them indirectly (non-enzymatically), with the likely mechanism being the production of metabolic products that act as reductants for Mn^{IV} such as oxalate (1, 103).

Phosphates

Phosphorus occurs primarily as organic phosphate esters and inorganic forms, e.g., calcium, aluminum, and iron phosphates. Organic phosphates are broken down by phosphatases which liberate orthophosphate during the microbial decomposition of organic material. Fungi also mobilize orthophosphate from insoluble inorganic phosphates by producing acids or chelators, e.g., gluconate, citrate, oxalate, and lactate, which complex the metal, resulting in dissociation. Phosphate-solubilization is very important in the plant mycorrhizosphere (110). Microbes can also play a role in the formation of phosphate minerals such as vivianite [$Fe_3(PO_4)_2.8H_2O$], strengite ($FePO_4.2H_2O$), and variscite ($AlPO_4.2H_2O$). The orthophosphate may be derived from organic phosphate degradation, while Fe or Al may arise from solubilization of other minerals. Such formation of phosphate minerals is probably most common in soil (1). Fungal biodeterioration of metallic lead can result in pyromorphite [$Pb_5(PO_4)_3X$ (X= F, Cl or OH)] formation (111–113). Many fungi can solubilize uranium oxides and depleted uranium and reprecipitate secondary uranium phosphate minerals, uramphite, and/or chernikovite, which can encrust fungal hyphae to high accumulation values (73, 74, 114). These minerals appear capable of long-term U retention (73, 74, 114, 115). *Aspergillus niger* and *P. javanicus* precipitated U-containing phosphate biominerals when grown with an organic P source with the hyphal matrix acting to localize the uranium minerals. The uranyl phosphates identified included potassium uranyl phosphate hydrate ($KPUO_6.3H_2O$), meta-ankoleite [$(K_{1.7}Ba_{0.2})(UO_2)_2(PO_4)_2.6H_2O$], uranyl phosphate hydrate [$(UO_2)_3(PO_4)_2.4H_2O$], meta-ankoleite [$K(UO_2)(PO_4).3H_2O$], uramphite ($NH_4UO_2PO_4.3H_2O$), and chernikovite [$(H_3O)_2(UO_2)_2(PO_4)_2.6H_2O$] (80). These organisms could also mediate lead bioprecipitation during growth on organic P substrates (81). These minerals were identified as pyromorphite [$Pb_5(PO_4)_3Cl$], which was only produced by *P. javanicus*, and lead oxalate (PbC_2O_4), which was produced by *A. niger* and *P. javanicus* (81). Several yeasts could also mediate lead bioprecipitation when utilizing an organic phosphorus-containing substrate (glycerol 2-phosphate, phytic acid) as the sole phosphorus source. The minerals precipitated here included lead phosphate [$Pb_3(PO_4)_2$], pyromorphite [$Pb_5(PO_4)_3Cl$], anglesite ($PbSO_4$), and the lead oxides massicot and litharge (PbO). All yeasts examined produced pyromorphite, and most produced anglesite (82).

Silicates

Silicates comprise 30% of all minerals and about 90% of the Earth's crust (116) (1, 60, 116). Many species of fungi play a role in the dissolution of silicates and therefore in the formation of clay minerals, and in soil and sediment formation (54, 87, 117–122). The presence of clay minerals can be a typical symptom of rock bioweathering by lichens and ectomycorrhizas (118, 119). Bioweathering is mainly indirect, through the production of metabolites together with biomechanical effects (123, 124). Geoactive metabolites may be excreted into the bulk phase but may also be produced by adhering organisms on silicate surfaces, resulting in etching (125, 126). After colonization of sheets of muscovite, a phyllosilicate mineral, by *A. niger*, dissolution was evident by a network of fungal "footprints" that reflected coverage by the mycelium (126). New biominerals resulted from fungal interactions with both zinc silicate and zinc sulfide, largely resulting from organic acid excretion. Zinc oxalate dihydrate was formed, and mineral surfaces showed varying patterns of bioweathering and biomineral formation (127). Silicate dissolution may release limiting nutrients such as bound P and Fe. In lichen bioweathering of silicates, calcium, potassium, iron, clay minerals, and nanocrystalline aluminous iron oxyhydroxides become mixed with fungal organic polymers (118), while biotite [$K(Mg,Fe(II))_3AlSi_3O_{10}(OH,O,F)_2$] was penetrated by fungal hyphae along cleavages, partially converting it to vermiculite [$(Mg,Fe(II),Al)_3(Al,Si)_4O_{10}(OH)_2.4H_2O$] (117). The fungal partner has also been reported to be involved in formation of secondary silicates such as opal ($SiO_2.nH_2O$) and forsterite (Mg_2SiO_4) in lichen thalli (128). The transformation rate of mica and chlorite to clay minerals was pronounced in ectomycorrhizosphere soil and was probably a result of production of organic acids and direct extraction of K^+ and Mg^{2+} by fungal hyphae (119). Fungal-clay mineral interactions also play an important role in soil development, aggregation, and stabilization (16, 129). Interaction between clay minerals and fungal biomass alters the sorptive properties of both clay minerals and fungal hyphae (130, 131) and also affects the size, shape, and structure of mycelial pellets (132).

Reduction or oxidation of metals and metalloids

Many fungi can precipitate reduced forms of metals and metalloids, e.g., Ag^{I} reduction to elemental silver Ag^{0}; selenate (Se^{VI}) and selenite (Se^{IV}) to elemental selenium (Se^{0}); and tellurite (Te^{IV}) to elemental tellurium (Te^{0}) (133–135). Reduction of Hg^{II} to volatile Hg^{0} can also be mediated by fungi (67, 68). Increased arsenate reduction contributed to tolerance in an *Aspergillus* sp. (136, 137). Mn oxidation/reduction is described above.

Other mycogenic minerals

A range of minerals other than those mentioned above have been found in association with fungi (2, 3, 73, 74, 77, 80–82, 114). Mycogenic secondary minerals associated with fungal hyphae and lichen thalli include desert varnish (MnO and FeO), ferrihydrite ($5Fe_2O_3.9H_2O$), iron gluconate, calcium formate, forsterite, goethite [α-$Fe^{3+}O(OH)$], moolooite [$Cu(C_2O_4).0.4H_2O$], halloysite [$Al_2Si_2O_5(OH)_4$], and hydrocerussite [$Pb_3(CO_3)_2(OH)_2$] (16, 48, 52, 108, 120, 128, 138). Another biogenic mineral (tepius) has been identified in association with a lichen carpet occurring in high mountain ranges in Venezuela (128).

Halide transformations

Several fungi have the ability to produce a variety of atmospheric methyl halides. This ability is widespread in both free-living and symbiotic fungi and is dependent on substrate concentration and community composition (139, 140). The production of chloromethane (CH_3Cl) by wood-rotting fungi, e.g., *Phellinus* spp., may be particularly significant, with one estimate of annual global input to the atmosphere from this source being 160,000 tons, of which 75% is released from tropical and subtropical forests (139). Filamentous fungi may also contribute to the global circulation of stable iodine and the long-lived radioiodine, ^{129}I (half-life: 1.6×10^7 years), released from nuclear facilities (141).

FUNGAL SYMBIOSES IN GEOMYCOLOGY

Many fungi form partnerships with plants (mycorrhizas) and algae or cyanobacteria (lichens) that are significant geoactive agents. In general terms, the mycobiont is provided with carbon by the photobionts, while the mycobiont may protect the symbiosis from harsh environmental conditions (e.g., desiccation, metal toxicity), and provide increased access to inorganic nutrients such as phosphate and essential metals.

Lichens are fungi that exist in facultative or obligate symbioses with one or more photosynthesizing partners occurring in almost all surface terrestrial environments (142). Lichens play important roles in retention and distribution of nutrient (e.g., C, N) and trace elements, in soil formation, and in rock bioweathering (54, 87, 143). Lichens can accumulate metals such as lead (Pb) and copper (Cu) and many other elements, including radionuclides (144). They also form a variety of metal-organic biominerals, e.g., oxalates, especially during growth on metal-rich substrates (98, 143). On copper sulfide-bearing rocks, precipitation of copper oxalate (moolooite) can occur within lichen thalli (145, 146).

The majority of terrestrial plants depend on symbiotic mycorrhizal fungi (147, 148). Mycorrhizal fungi can mediate metal and phosphate solubilization from mineral sources, effect extracellular precipitation of metal oxalates, and immobilize metals within biomass (65, 66, 149–157). Such activities lead to changes in the physico-chemical characteristics of the root environment and enhanced bioweathering of soil minerals (55, 157, 158). Furthermore, ectomycorrhizal mycelia may respond to different soil silicate and phosphate minerals (e.g., apatite, quartz, potassium feldspar) by regulating growth and metabolic activity (159, 160).

Mycorrhizal fungi often excrete bioweathering agents such as low–molecular-weight carboxylic acids and siderophores (65, 161). Ectomycorrhizal fungi can also form narrow pores in weatherable minerals in podzol E horizons, probably by dissolution of Al silicates (162, 163). Such excretions can also release elements from apatite and wood ash (K, Ca, Ti, Mn, Pb) (164). Ericoid mycorrhizal and ectomycorrhizal fungi can dissolve several cadmium, copper, zinc, and lead-bearing minerals including metal phosphates (65, 66, 152, 161, 165). Mobilization of phosphorus from inorganic and organic phosphorus sources is generally regarded as one of the most important functions of mycorrhizal fungi, and this can also result in redistribution of incorporated metals and the formation of other secondary minerals including other metal phosphates. The ericoid mycorrhiza *Oidiodendron maius* can solubilize zinc oxide and phosphate (161). Many ericoid mycorrhizal and ectomycorrhizal fungi are able to solubilize zinc, cadmium, copper phosphates, and lead chlorophosphate (pyromorphite), releasing phosphate and component metals (65, 152). An association of arbuscular mycorrhizal fungi with *Lindenbergia philippensis*, sampled from a Zn-contaminated settling pond at a zinc smelter, enhanced Zn accumulation in Zn-loaded rhizosphere sediment compared to treatments that suppressed arbuscular mycorrhizal fungi colonization. A significant proportion of Zn was present as crystalline and other solid materials that were associated with the root

mucilaginous sheath (166). Such results may indicate a role for arbuscular mycorrhizal fungi in enhancing Zn immobilization in the rhizosphere of plants that successfully colonize Zn mining and smelting disposal sites (153, 166–168).

ENVIRONMENTAL AND APPLIED SIGNIFICANCE OF GEOMYCOLOGY

The kinds of processes detailed previously can impact human society not only through their environmental significance and biotechnological applications, but also in deleterious contexts such as biodeterioration and biocorrosion. The biodeterioration of stone and mineral artifacts represents a loss of cultural heritage (13, 14). Materials used to stabilize building blocks (mortar) and to coat surfaces prior to painting (plaster or stucco) are also susceptible to biodeterioration (13). Highly deteriorated stone surfaces provide a "protosoil" for colonization by mosses, ferns, and higher plants (14). Mechanisms of stone deterioration are complex and include most of the direct and indirect mechanisms previously discussed for mineral dissolution (13, 169). Extracellular polymeric substances are also capable of metal complexation and weakening of mineral lattices through wetting and drying cycles, as well as the production of efflorescences, i.e., secondary minerals produced through the reaction of anions from excreted acids with cations from the stone (170). Physical damage may be caused by hyphal penetration of weakened areas (88, 138). Lichens cause damage due to penetration by their rhizines, composed of fungal filaments, and expansion/contraction of the thallus on wetting/drying (171). "Lichen acids," mainly oxalic acid, cause damage at the stone-lichen interface, and lichen thalli may accumulate up to 50% calcium oxalate, depending on the substrate (172, 173). In addition, carbonic acid formed in the lichen thallus can solubilize calcium and magnesium carbonates in calcareous stone (174). Fungal biodeterioration of ancient ivory (natural apatite; walrus tusk) was accompanied by widespread etching and tunneling by hyphae and extensive formation of calcium oxalate monohydrate, whewellite (175). Concrete and cement can be biodeteriorated, and in some environments, fungi dominate the concrete-deteriorating microbiota (13, 14, 176–178). Microbial attack on concrete is mediated by protons, inorganic and organic acids, and the production of hydrophilic slimes leading to biochemical and biomechanical deterioration (13, 75, 169). Several species of microfungi were able to colonize samples of the concrete used as a radioactive waste barrier in the Chernobyl reactor and leached iron, aluminum, silicon, and calcium and reprecipitated silicon and calcium oxalate (75).

Mineral and metal solubilization mechanisms enable metal removal from industrial wastes, low-grade ores, and metal-bearing minerals. This may have applications in bioremediation, metal biorecovery, and recycling (58, 68, 179, 180). Metals can be solubilized from fly ash (originating from municipal solid waste incineration), contaminated soil, electronic scrap, and other waste materials by fungal activity (179, 181). Although fungal systems cannot compare with the efficiency of bacterial bioleaching, they may be more suited to specific bioreactor applications (58). A variety of fungal mechanisms result in metal immobilization such as biosorption, bioaccumulation, and bioprecipitation. Biosorption is a physico-chemical process and is a property of both living and dead organisms (and their components), and fungi are effective agents for removal of metals, radionuclides, and other substances from solution (69–71, 76, 130, 182–193). Urease-positive fungi can be used to precipitate metal-containing carbonates, some in nanoscale dimensions, thus providing a means of metal biorecovery as well as potentially useful nanoscale biomineral products (94, 95). Similarly, the formation of other insoluble metal compounds by fungi or their metabolites could also be considered as a means to biorecover metals, metalloids, and radionuclides, e.g., oxalates, oxides, and phosphates, as well as the production of elemental metal or metalloid forms (2, 78). Some biomineral and elemental products, including those of nanoscale dimensions, are relevant to the production of novel advanced biomaterials, with applications in metal and radionuclide bioremediation, antimicrobial treatments (e.g., nano-silver), solar energy and electrical battery applications, and microelectronics (194). In a novel approach, urease-positive *N. crassa* was used to precipitate manganese carbonate. After thermal treatment at 300°C, the carbonized biomass-manganese oxide composite material was used in lithium-ion batteries and supercapacitors, where it was found to exhibit excellent electrochemical properties. In lithium-ion batteries, around 90% charge capacity was retained after 200 charge-discharge cycles (195).

The ability of fungi and bacteria to transform metalloids has been successfully used for bioremediation of contaminated land and water. Selenium methylation results in volatilization, and this has been used to remove selenium from the San Joaquin Valley and Kesterson Reservoir, California (196). Mycorrhizal associations may have applications in phytoremediation (197, 198), the use of plants to remove or detoxify

environmental pollutants (199), by metal phytoextraction or by acting as a biological barrier (200–202). Glomalin, an insoluble glycoprotein, is produced in copious amounts on hyphae of arbuscular mycorrhizal fungi and can sequester metals such as Cu, Cd, and Pb (203). Arbuscular mycorrhizal fungi can also decrease U translocation from plant roots to shoots (204–206). For ericaceous mycorrhizas, the fungus prevents translocation of Cu and Zn to host plant shoots (147, 207, 208). The development of stress-tolerant plant-mycorrhizal associations may be a promising strategy for phytoremediation and soil amelioration (161, 209, 210).

Some of the geomycological processes detailed previously may have consequences for abiotic soil treatment processes, notably the immobilization of toxic metals by phosphate formation. Apatite [$Ca_5(PO_4)_3$(F, Cl,OH)], pyromorphite [$Pb_5(PO_4)_3Cl$], mimetite [$Pb_5(AsO_4)_3Cl$], and vanadinite [$Pb_5(VO_4)_3Cl$] are the most common prototypes of the apatite mineral family. Such minerals hold promise for stabilization and recycling of industrial and nuclear waste and have been explored for treatment of lead-contaminated soils and waters (211–216). The stability of these minerals is therefore of interest in any soil remediation strategy seeking to reduce the effects of potentially toxic elements such as Pb, V, and As. For example, pyromorphite is a highly insoluble lead phosphate mineral under a wide range of geochemical conditions and has often been suggested as a means to reduce Pb bioavailability. However, solubilization of pyromorphite and formation of lead oxalate by several free-living and symbiotic fungi demonstrate that pyromorphite may not be as effective at immobilizing lead as some previous studies have suggested (64, 65). Similarly, despite the insolubility of vanadinite, fungi exerted both biochemical and biophysical effects on the mineral including etching, penetration, and the formation of new biominerals (217). Lead oxalate was precipitated by *A. niger* during the bioleaching of vanadinite and mimetite, which suggests a general fungal mechanism for the transformation of lead-containing apatite-group minerals (e.g., vanadinite, pyromorphite, mimetite) (217, 218).

This pattern of fungal bioweathering of lead apatites could be extended to other metal apatites, such as calcium apatite [$Ca_5(PO_4)_3(OH,F,Cl)$]. Here, the formation of monohydrated (whewellite) and dihydrated (weddellite) calcium oxalate can be accomplished by many different fungal species (79, 93, 99, 175, 219, 220). The ability of free-living and mycorrhizal fungi to transform toxic metal-containing minerals should therefore be taken into account in risk assessments of the long-term environmental consequences of *in situ* chemical remediation techniques, revegetation strategies, or natural attenuation of contaminated sites. The bioweathering potential of fungi has been suggested as a possible means for the bioremediation of asbestos-rich soils. Several fungi could extract iron from asbestos mineral fibers (e.g., 7.3% from crocidolite and 33.6% from chrysotile by a *Verticillium* sp.), thereby removing the reactive iron ions responsible for DNA damage (221).

CONCLUSIONS

The geoactive roles of fungi have often received scant attention in geomicrobiological contexts, but they are of clear importance in several key areas. These include a variety of organic and inorganic transformations important in nutrient and element cycling, rock and mineral bioweathering, mycogenic biomineral formation, and metal-fungal interactions. Lichens and mycorrhizas are of special significance as geoactive agents. Organic matter decomposition is important for the cycling of major biomass-associated elements, e.g., C, H, N, O, P, and S as well as all other elements that may be found in lower concentrations. Transformations of metals and minerals are central to many geomicrobial processes, and fungi can effect changes in metal speciation, toxicity, and mobility, as well as mediate mineral formation or dissolution. Such mechanisms are important components of natural biogeochemical cycles for metals as well as associated elements in biomass, soil, rocks, and minerals, e.g., S and P, and metalloids, actinides, and metal radionuclides. It is within the terrestrial environment where fungi have the greatest abundance and geochemical influence. However, they are also important in aquatic habitats and are now recognized as significant components of aquatic sediments and the deep subsurface. Geomycological processes can have beneficial or detrimental consequences in a human context. Beneficial applications in environmental biotechnology include metal and radionuclide bioleaching, biorecovery, detoxification, and bioremediation, and in the production of biominerals or metal(loid) elements with catalytic or other properties in nanoparticle, crystalline or colloidal forms. The latter may be relevant to the development of novel biomaterials. Adverse effects include biodeterioration and destruction of natural and synthetic materials; rock and mineral-based building materials (e.g., concrete); cultural heritage; biocorrosion of metals, alloys, and related substances; and adverse effects on radionuclide speciation, mobility, and containment. The ubiquity and importance of fungi in biosphere processes underlines the importance of

geomycology as a conceptual framework encompassing the environmental activities of fungi, their impact, and their applied significance.

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Ecology of Fungal Plant Pathogens

17

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INTRODUCTION

Plant pathogens are parasites that live at the expense of their host. While fungal pathogens are the largest group of plant pathogens, other important plant pathogens include bacteria, protists, chromists, nematodes, and even plants. Although this wide variety of pathogens share many aspects in epidemiology and management, here we deal only with fungal plant pathogens. The economic importance of fungal plant pathogens in the production of food, feed, materials, and ornamentals is undisputed (1). Direct costs include yield loss and use of resistant cultivars or pesticides. Indirect costs include the inability to grow certain crops or cultivars at a given location. Inspection and quarantine protocols to prevent the dispersal of pathogens (2) are indirect costs that are rarely taken into account. In contrast, as will be shown in this review, plant pathogens in nature are regarded as crucial contributors to the maintenance of biodiversity, similar to the role major animal predators play in wildlife.

Plant pathogens comprise a heterogeneous group of organisms that share the ability to infect their hosts. Pathogens able to infect apparently healthy plants are referred to as primary pathogens. Secondary pathogens typically appear after infection by a primary pathogen. Weak or minor pathogens usually cause negligible yield decline, although stronger yield loss can result if the host is stressed by other biotic or abiotic factors. The remarkable heterogeneity of plant pathogens can be expressed in multiple ways (3):

- **Nutrition:** Biotrophic (e.g., powdery mildews and rust fungi) or necrotrophic (e.g., most leaf spot diseases).
- **Host range:** From polyphagous (e.g., *Botrytis cinerea*) to plant species- or cultivar-specific (e.g., *Puccinia graminis* causing black rust on wheat).
- **Survival:** Inside living host tissue or on crop debris and in soil.
- **Infection:** Through stomata (e.g., rust fungi), direct penetration (e.g., many leaf spot diseases and smut diseases) or through wounds (many wood-rotting fungi).
- **Dispersal:** By wind (long range, e.g., many airborne pathogens, water-splash (short range, e.g., *Cercospora* leaf spot), growth in soil (short range soil-borne pathogens), or vectors, mainly insects (mid- to long-range, e.g., *Ophiostoma novo-ulmi*); for agronomic plant pathogens, anthropogenic dispersal is probably most important (infested soil and infected plants including seeds).
- **Symptoms:** E.g., ongoing necrosis of stem (stem blight) or leaves (leaf blight), limited necrosis development (leaf spot or anthracnose), wilting, seed or flower infection, root rot, seedling damping-off.
- **Taxonomic position:** Chytridiomycota (mostly pathogens of algae), Ascomycota (containing most of the fungal plant pathogens) and Basidiomycota (mostly wood rot-causing fungi but also includes the important pathogen *Rhizoctonia solani* [syn. *Thanatephorus cucumeris*]). Except for some post-harvest diseases, the Zygomycota are of lesser importance.
- **Persistence:** Variable longevity (from days to years); typically, soil-borne pathogens are persistent, with a minimal survival time of 1 year.
- **Plant-pathogen interaction:** Monogenic gene-for-gene relation or polygenic.
- **Activity inside the host:** Remaining local (many leaf spot fungi) to having the ability to move within the plant through systemic growth (e.g., powdery mildews) or through xylem tissue (wilt fungi).
- **Life cycle:** Monocyclic or polycyclic (i.e., referring to the number of generations within one growing season); with or without a sexual phase, with or without host alternation.

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Knowledge of these attributes of plant pathogens provides keys to their management. Examples include the following:

- Obligate pathogens often tend to respond strongly to host nutritional status, which can be influenced by fertilization management.
- Pathogen host range provides information on crops that can be cultivated.
- Site of pathogen survival determines what plant parts (e.g., cuttings) can be safely transported.
- Pathogen dispersal strategy impacts the need for forecasting systems.
- Disease symptomatology is needed for proper pathogen recognition.
- Pathogen persistence provides information on the time a farmer has to refrain from cropping a host on the infested soil.
- Host relationship indicates the perspectives of resistance breeding.
- Pathogen activity within the host predicts disease development within a host.
- Pathogen life cycle attributes predict epidemic disease development.

Because plant pathogens threaten the world's food security, most research has been done on pathosystems in arable and greenhouse farming. In addition, they infect a range of ornamental crops of great economic value. Only recently has the significance of pathogens in natural ecosystems been appreciated (4).

PLANT DISEASE

Plant disease is an abnormal behavior of plant development. What abnormal plant development actually is can be a semantic discussion (5), but in its extreme, i.e., heavily attacked plants which die prematurely, it is obvious. On the other hand, a minor infection of, say, a few pustules per plant of a rust pathogen, is practically harmless but can be the initial stage of a major epidemic. Plant diseases may remain undetected because the symptoms they produce are invisible or indistinguishable from other factors. For example, in potato *Verticillium dahliae* incites the crop to die a few weeks earlier than would be the case in its absence, which often goes unnoticed by the farmer (6). In agriculture, a pragmatic approach is that a disease is only deemed significant if the pathogen affects the marketable yield.

The classic conceptual framework for evaluating disease is the so-called disease triangle (7). Although many variants of the original disease triangle have been published (e.g., reference 8), they all essentially state that plant disease depends on the characteristics of host, pathogen, and environment. Thus, the minimum requirement for plant disease to develop is the presence of a virulent pathogen, a susceptible host, and suitable biotic and abiotic environmental conditions. The outcome of this interaction (plant disease and potentially a subsequent epidemic) depends on quantitative characteristics such as the amount of pathogen and host present, the rate of pathogen and host development as a function of environmental conditions, and the sensitivity of the host. An interesting example of how the disease triangle could be used in predicting the coexistence of plant biodiversity is provided by Mordecai (9).

Disease Triangle Elements: Plant Pathogen

Pathogens are classified primarily on the basis of their genus and species name. Pathogens often appear more variable than initially assumed. For example, the great majority of *Fusarium oxysporum* organisms occurring in soil are not plant pathogenic, and only a minority are able to infect root tissue. Morphological differences between pathogenic and nonpathogenic strains are absent, and pathogenicity toward a single host can be polyphyletic (10, 11). The same is true for *R. solani*, which is differentiated into anastomosis groups on the basis of vegetative incompatibility of agar-cultivated mycelia. These anastomosis groups have been differentiated into subgroups and sub-subgroups which, respectively, show some and limited vegetative incompatibility (12). These subdivisions appear functional because they exhibit different ecological traits in, e.g., host range and temperature characteristics. In some cases, the inability to anastomose in pure culture has led to improved insight into speciation (e.g., intersterility groups in the *Armillaria mellea* species complex [13]), whereas in other cases this is much less the case (e.g., vegetative compatibility groups in *V. dahliae* [14]). For *Synchytrium endobioticum*, the causal agent of potato wart, a range of pathotypes exist that can only be differentiated on the basis of bioassays (15). All these infraspecific rankings are confusing, and therefore a call has been made to include sequence data in all research and publications and to infer from this information the genetic basis and characteristics of the particular strain being studied (16, 17). While this approach is needed for improving our knowledge of pathogens, their accurate scientific names are essential for efficient communication among a variety of stakeholders such as farmers, their advisors, and policy-makers.

Pathogens may interact with each other. For example, synergistic effects between wilt fungi (*V. dahliae* and *F. oxysporum*) and plant parasitic nematodes

(*Pratylenchus* and *Meloidogyne*) are known (18). The mechanism of this fungus-nematode interaction is not well understood. The nematode may provide infection courts for the wilt fungus, but spatial separation of the fungus and nematode also aggravates disease (19). Leguminous crops are well known for the consortia of fungi (including oomycota) that cause root rot (20, 21).

Pathogen density, along with host density (see below), plays a crucial role in the development of an epidemic. When host density in a given crop does not vary, pathogen density is of great importance to predict disease caused by soil-borne pathogens. Therefore, preplant soil inoculum densities can be used to decide the crop or cultivar choice (e.g., reference 22).

Disease Triangle Elements: Host

Host density plays a prominent role in the epidemic development of a disease. For several diseases, a lower-threshold host density for dispersal has been defined (23, 24). For example, attempts to establish plantations of *Hevea brasiliensis*, the source of natural rubber, within its native range have largely failed due to epidemic occurrence of rubber leaf blight caused by *Pseudocercospora ulei* (syn. *Microcyclus ulei*). This pathogen is only of minor importance at natural sites of the rubber tree, and the difference within plantations has been attributed to the unnaturally high density of the host in plantations (25). By and large, this is true for many of the pathogens that are important in agroecosystems, where they cause greater damage due to high host density than in plant species-rich natural vegetation (26). The level and type of resistance of the host plant is another decisive factor in disease development. For instance, the threshold inoculum level of *V. dahliae* for cotton was estimated at 4 and 7 CFU per gram of soil for susceptible and resistance cultivars, respectively (22).

Disease Triangle Elements: Environment

The environment comprises the whole array of biotic and abiotic conditions that define host susceptibility and pathogen status. Soil type and nutrition play important roles in the response of the host to a pathogen. A shortage of essential macro- or micronutrients may lead to increased susceptibility (27); in particular, ammonium and nitrate nutrition can interact with certain obligate pathogens (28). Tropical pathogens can be frost-sensitive (e.g., *Macrophomina phaseolina* and *Sclerotium rolfsii*), while others are inactive at high temperatures that prevail in the (sub)tropics (e.g., *V. dahliae*). Certain biota are able to suppress pathogens, e.g., yeast fungi in the phyllosphere (29) and certain bacteria and fungi in the soil (30). Abiotic stress leading to increased susceptibility or resistance of the host to later infection is referred to as predisposition (reviewed in reference 31). An example is the off-site planting of *Cupressus macrocarpa*, where cypress canker, caused by *Seiridium cardinale*, causes much damage (32). The host grows well in these off-site, relatively dry conditions in the absence of the pathogen, but in the pathogen's presence it suffers much damage, whereas at its native site, more humid and cool, the pathogen is virtually absent. This differential effect may reflect the allocation of resources to growth at the expense of defense actions (33). Abiotic factors enhancing pathogens under certain conditions enhance predisposition. It has been suggested that tree seedlings that typically grow in dry habitats cannot do so in wet conditions, due to their sensitivity to soil-borne pathogens that thrive well in those conditions (34).

Three major groups of plant pathogens are usually recognized; soil-, air-, and seed-borne plant pathogens. Soil-borne pathogens are able to survive in soil for at least 1 year but usually for multiple years in the absence of a host (35). Only a few soil-borne pathogens sporulate aboveground, so the majority of them have limited dispersal capability (but see reference 36 for some exceptions). Airborne pathogens are not able to survive a cropless period within soil. They either survive on organic debris situated on the soil (e.g., grain stubble) or, in the case of perennial hosts, inside the living host. If they survive consistently within seeds, they are generally referred to as seed-borne fungi. These traits have major consequences for their management in agroecosystems (Table 1): inocula of airborne pathogens on crop debris incorporated into the soil will be inactivated quickly. Predicting the occurrence of epidemics of airborne pathogens is difficult because of their long-range dispersal. Fungicides may have strong effects on airborne pathogens, but in soil they are decomposed quickly by the soil microbial community, rendering many of them ineffective. Resistance genes are available for airborne pathogens but much less so, or not at all, for soil-borne pathogens. Predicting disease caused by soil-borne pathogens is based on the estimation of soil inoculum density. Such tests are usually costly, especially if spatial distribution (which very often is patchy) is to be taken into account. Soil-borne pathogens can be managed using proper rotations, while this is not the case for airborne pathogens. The primary focus of managing seed-borne pathogens is to produce uninfected seeds and to carry out effective seed treatment to clean infected seeds.

These three major groups of soil-, air- and seed-borne pathogens do share specific characteristics. Host range

Table 1 Characteristics of the three major ecological groups of fungal plant pathogens[a]

Characteristic	Soil-borne pathogens	Airborne pathogens	Seed-borne pathogens
Survival	In soil; often multiple years in the absence of a host	On infected crop debris for a relatively short duration, in seeds, or in perennial hosts	Within seeds
Dispersal	Natural dispersal short-range, slow, usually by root-to-root contact but also by airborne soil dust (dust storms); anthropogenic dispersal by moving infested soil and infected planting material	Natural dispersal long-range, fast, by massive spore production that is transported by air or by splash; anthropogenic dispersal by infected planting material	By infected seeds; subsequent sporulation on infected plants
Disease cycle	Usually monocyclic	Usually polycyclic	Mono- or polycyclic[b]
Key epidemiological parameters	Soil inoculum density, persistence	Climate models, mode of dispersal (wind- or splash-transmitted)	Quantity of seed infection, presence of sporulation on infected plants[b]
Management	Crop rotation, soil quality improvement	Pesticides, resistant cultivars, disease forecasting	Seed disinfestation, pesticides, use of uninfected seeds

[a]Not all pathogens fit into this classification. For example, *Sclerotinia sclerotiorum* and several smut fungi are highly persistent in soil and have a phase of airborne dispersal of spores as well.
[b]Many seed-borne pathogens have an airborne phase in which they are polycyclic.

in these groups varies from very small to very wide. For example, a small host range occurs in the soil-borne potato wart disease caused by *S. endobioticum*, which infects only potatoes, and in many airborne powdery mildew fungi and seed-borne smut fungi that have only a single host plant. Very wide host ranges are exemplified by the soil-borne *Verticillium* wilt caused by *V. dahliae*, which is able to infect hundreds of hosts, and the airborne *B. cinerea*, which is able to cause all kinds of shoot, flower, and fruit rot in many plant species.

LATENT INFECTIONS AND ENDOPHYTES

One of the basic elements of plant pathogen disease cycles is their latency period, which is defined as the time between infection and the appearance of the first disease symptoms (3). Infections before symptom development are usually only detected by isolating the pathogen. In international trade, such latency periods can be the reason for keeping suspected material in quarantine for some time. However, also after the appearance of the first disease symptoms pathogens can move into an asymptomatic, latent stage. Examples include the survival of pathogens in seeds or in overwintering buds of trees. Latent infections impede the inspection of plant health since they are easily overlooked.

Some pathogens are also able to infect hosts without causing any disease symptoms, whereas in others they readily cause disease. For example, Malcolm et al. (37) reported symptomless infection of *V. dahliae* (a primary wilt pathogen of, i.e., olive, strawberry, and cotton) in 9 agronomic crops and 25 weed species. Plant-infecting fungi not causing disease symptoms are referred to as endophytes (38, 39). Thus, whether *V. dahliae* should be classified as pathogen or endophyte is host-dependent. This endophytic behavior of a primary pathogen in other hosts makes inspection procedures difficult. To complicate matters further, *V. dahliae* is known to have two types of latent stage: first, the infection of root systems and, second, the interruption of wilt development at temperatures above 30°C (40). Despite this variable behavior, *V. dahliae* is still regarded as a single species (16). Some important widespread pathogens appear to be clonal in nature (e.g., Panama disease of banana caused by *F. oxysporum* f. sp. *cubense* race 4 [41]), but in general, the adage is "Show me a plant pathogen, and I will show you a species complex" (42). For example, *Colletotrichum acutatum* appeared to consist of 31 taxa (43). Failure to scout diseased strawberry plants infected by *C. acutatum* (44), the causal agent of anthracnose, due to its latent presence caused large economic losses in many places (45). Other examples of latent pathogens are provided by Crous et al. (17).

EFFECTS OF FUNGAL PLANT PATHOGENS

The dilution effect hypothesis predicts a lower abundance of pathogens if their hosts occur in biodiverse

plant communities (46). There is a vast body of data supporting this conclusion (46). For example, the leaf area infected by fungal pathogens was on average 3 times higher in single-species grasslands than in a mixed grassland containing 24 planted species (47), and the number of host-specific leaf pathogens of trees is fewer in mixed forests (48). The magnitude of the dilution effect depends on the phylogenetic composition of the vegetation and the host range of the plant pathogens (49). The main hypothesis explaining the dilution effect hypothesis is host camouflage: pathogens have a lower probability of arriving at a host's surface if more nonhosts are present, because nonhosts intercept airborne propagules or because host-specific signals are diluted. An additional mechanism could be differential behavior of auto-infections (i.e., infections arising from inoculum produced on the same plant) compared to allo-infections: auto-infections of wheat by *Puccinia triticina* lead to shorter latent periods (i.e., the period from infection to producing new inoculum) than allo-infections (50). Irrespective of the outcome of a host-pathogen interaction, that is, disappearance of host or pathogen or coexistence, Salama et al. (51) argued that in a natural ecosystem the magnitude of the seed bank of the host would dampen these effects.

The Janzen-Connell hypothesis predicts that host-specialized pathogens maintain high diversity by elevating mortality when plant species occur at high density; i.e., if more host plants exist, the pathogen causes greater disease and the number of host plants is reduced (52, 53). More specifically, this hypothesis has been used to explain the high tree species biodiversity in tropical forest ecosystems. It is now widely accepted that natural enemies, including fungal pathogens, play a major role in this. For example, in the tropical rainforests of Belize, the negative density dependence of seedling mortality was attenuated after application of broad-spectrum fungicides (54). In a more detailed study, the low germination of seedlings of *Ormosia glaberrima* in forests with a high density of mature specimens of this species was reversed by fungicide treatment (55). The authors suggested that *F. oxysporum* could have been the cause of this. Where pathogens limit the development of certain plant species, they leave space for other species, thus promoting vegetation diversity in space (56) or in time (57).

In forests, canopy gaps are crucial elements for forest dynamics and diversity (58). In unmanaged *Pinus mugo* forests, canopy gaps appeared to be heavily infested with *Heterobasidion annosum* and *Armillaria ostoyae*, suggesting that they were the causal factors of these gaps (59). A range of root pathogens can occur in and on the fine roots of older trees, as was observed by Chavarriaga et al. (60) for seminatural and planted *Pinus sylvestris* in Scotland. Pathogen enrichment in soils underneath older trees reducing the growth of conspecific seedlings has been observed in a seminatural forest for seven out of eight tree species studied (61). Similarly, Packer and Clay (62) reported that fungus-like *Pythium* spp. isolated from mature *Prunus serotina* incited mortality of conspecific seedlings, and they suggested that this explained the usually large distance between trees in their native range in the United States. Burdon et al. (63) mentioned other examples of this intriguing phenomenon. It would be interesting to see if this hypothesis could be falsified for vegetation that has reached its final successional stages, such as beech forest. The Janzen-Connell hypothesis was formulated for narrow host range pathogens (52, 53). However similar mechanisms for wide host range pathogens can also be applicable, because their quantitative effects on plant growth and development are usually strongly host-species-dependent (64) and may also be shaped by differential effects of the environment on the host-pathogen relationship (9).

Clearly, the dilution effect and the Janzen-Connell hypothesis are closely linked, with the focus of the first more on host density, and the second on pathogen (and pest) enrichment. Both hypotheses originate from work on natural ecosystems, but agroecosystems fit well in the picture. The Janzen-Connell hypothesis is well illustrated by the fruit tree replant disease (65, 66): a variety of pathogens present on the relatively large root systems of older trees successfully attack the small root systems of younger, recently planted trees, because they are likely stressed shortly after planting. Gilligan and coworkers showed that threshold densities of hosts were needed to achieve focus expansion by the soil-borne pathogen *R. solani* (24). The same idea applies for the time dimension. The majority of soil-borne pathogens increase their population size if more hosts in the rotation are included (67). For example, problematic enrichment of a multitude of pathogens has been demonstrated for the perennial grass *Ammophila arenaria* planted to stabilize coastal sand dunes. Under natural conditions, these pathogens have been shown to accelerate vegetation succession (68). Similarly, pathogen enrichment of *F. oxysporum* f. sp. *asparagi* was shown to be a common phenomenon in the cultivation of asparagus, shortening its cultivation by several years. Because of the extreme persistence of the pathogen, once infested, a field was rendered unproductive for this crop for decades (69). In its most simple form, arable farming systems exemplify the dilution effect

hypothesis, where diseases generally are more prominent if more hosts per unit of area are cultivated (70). Lowering host density by introducing intercropping, mixed cropping, or widening rotations could reduce these problems, but these methods usually face significant practical difficulties with mechanized crop treatments such as sowing and harvesting.

Host-specific pathogens may play a role in the development of host root density. Evidence of this is suggested by the repeatedly reported overyielding of root biomass in species mixtures compared to their respective monocultures (71, 72). This can be seen as a special case of the dilution effect hypothesis. After soil sterilization or fungicide treatment, root biomass in monocultures increases to levels similar to that of the plant mixtures (73, 74). Also, pathogen-suppressive microorganisms enriched in specific rhizospheres may play a role. This has been observed repeatedly; specifically, plant growth is less constrained if the soil is enriched with rhizosphere microorganisms from other plant species rather than from its own rhizosphere (reviewed in reference 72).

Although high host density creates good conditions for pathogen build-up, pathogen distribution is often highly erratic. The dispersal of airborne pathogens depends strongly on climatic conditions, and dispersal of soil-borne pathogens is often facilitated by using infected planting material or machines with infested soil adhering to them. The degree to which pathogens shape vegetation composition may vary, because other conditions can be decisive as well. For example, McCarthy-Neumann and Kobe (75) determined that shading is an important factor for explaining vegetation biodiversity. Kos et al. (76) discovered that distribution of *Jacobaea vulgaris* was explained by local nutrient availability and seed bank density.

BIOINVASIONS OF FUNGAL PLANT PATHOGENS

Invasion of pathogens in new areas (bioinvasions) are often mediated by humans and are the cause of major disease outbreaks. In newly invaded regions, hosts and pathogens may not have coevolved, resulting in high host susceptibility and dramatic epidemics. For example, the Dutch elm (*Ulmus* spp.) disease, caused by *Ophiostoma ulmi* and later by *O. novo-ulmi*, was transported by unknown means from Central Asia to Europe, where elms were destroyed, and later elsewhere by human transportation (77). Karnal bunt on wheat, a smut disease caused by *Tilletia indica*, has been dispersed through anthropogenic means from India to Mexico and the United States. Though the disease does not cause much damage, its further dispersal is unwanted because of its extremely high persistence, and therefore it inflicts high costs because of export constrictions (78). Another recent example is the outbreak of ash (*Fraxinus*) dieback, which is caused by two related, initially spatially separate strains of *Hymenoscyphus*. After sexual crossing, a highly virulent new species, *Hymenoscyphus fraxineus*, evolved, which now is causing a major epidemic in European ash trees (79). New arrivals may cause considerable damage for some time, but eventually natural enemies may appear, such as viruses infecting these fungal pathogens (80, 81), as has been shown for chestnut blight of *Castanea sativa* caused by *Cryphonectria parasitica* (82) in Europe. Bioinvasions are also mediated by changes in the environment, such as climate change (reviewed by Bebber [83]). The majority of agronomic diseases are examples of bioinvasions, because many crops, and therefore their pathogens, are grown outside their natural distribution area. More generally, novel diseases may arise from several sources: coevolution during domestication, pathogen hybridization, host shift and host jump, and horizontal gene transfer (84, 85).

Coevolution during Domestication

Stukenbrock et al. (84) have provided examples of coevolution during domestication, such as rice blast caused by *Pyricularia oryzae*. Coevolution leads to more specialization: nonrice strains appeared less pathogenic on rice (86). If this is a common phenomenon, then it would predict reduced effects of these pathogens on natural vegetation. Remarkably, the topic of how agronomic pathogens shape natural ecosystems has barely been addressed. Their effects may be limited: in the many examples of pathogens inflicting damage in natural vegetation, Gilbert (87) did not mention major agronomic pathogens.

Pathogen Hybridization

Infection by powdery mildew (*Blumeria graminis* f. sp. *triticale*) on triticale, an artificial hybrid of wheat and rye, has been known only since 2001. It recently became clear that this pathogen originated after hybridization of *B. graminis* f. sp. *tritici* on wheat and *B. graminis* f. sp. *secalis* on rye (88). Hybrids between *O. ulmi* and *O. novo-ulmi* have been recorded (89), and although they appear less aggressive than their parents, they may eventually lead to increased pathogenicity (84). The diploid *Verticillium longisporum* appears to be the result of multiple hybridization events

of an unknown *Verticillium* species with isolates of *V. dahliae* (90). *V. longisporum* attacks brassicaceous hosts, in contrast to the large majority of *V. dahliae* isolates. The above-described recent outbreak of ash dieback is probably also the result of hybridization. With the increased worldwide movement of pathogens due to trade, occurrence of hybridization is likely to become more important (91).

Host Shift

Host shifts occur when a pathogen invades phylogenetically close host relatives. An example is *C. parasitica*, the causal agent of chestnut blight, which coexists with the host in Eurasia but which causes extensive tree death in North America. This is thought to be due to the lack of coevolution and hence high virulence of the pathogen (92). Another host shift was assumed for *R. solani* anastomosis group 1 IA, from rice to signal grass (*Urochlora* sp.) in Colombia and Brazil (93). Host shifts of tree-pathogenic members of the Ophiostomatales were assessed to be significant for phylogenetically closely related hosts when wood-boring beetle vectors are present (94).

Host Jump

Host jumps occur when a pathogen infects a phylogenetically distant host. Host jumps are likely to occur for many soil-borne pathogens, for which vertical, monogenic resistance is the exception rather than the rule. The soil-borne wilt-causing and now cosmopolitan *V. dahliae* appears to exhibit little spatial differentiation in genetic structure, and global invasion through anthropogenic distribution of infested soil or planting material seems the best explanation (95), yet its host range is massive (96), and many host jumps must have occurred.

Horizontal Gene Transfer

Transfer of the *ToxA* gene from *Parastagonospora nodorum* (syn. *Phaeosphaeria nodorum*) (which causes wheat blotch) into *Pyrenophora tritici-repentis* (which causes tan spot in wheat) has been described (97). The limited number of interspecific polymorphisms in both coding and noncoding DNA suggest that this transfer occurred recently and is consistent with the fact that tan spot was observed for the first time in 1941 (97). Also, toxins of pathotypes of *Alternaria alternata* have likely been acquired by horizontal gene transfer (98). Although horizontal gene transfer seems to occur only sporadically, it is clear that once it has occurred, the damage may be quite large, as exemplified by *P. tritici-repentis*, which is now distributed worldwide.

Many records of novel bioinvasive diseases are due to the introduction of pathogens outside their native range. The immediate danger of this is host jumps and host shifts, but rare events such as pathogen hybridization and horizontal gene transfer are important as well. Stukenbrock and McDonald (92) argued that agriculture may increase the frequency of these events compared to natural ecosystems, because pathogens on crops occur in high densities and, consequently, in vast amounts.

Fungal pathogens play an important role in suppressing the dynamics of their hosts. Being bioinvasive weeds elsewhere, in their native range *Centaurea maculosa* (spotted knapweed) (99), *P. serotina* (57, 100), and *A. arenaria* (101) are maintained at low densities because of the presence of root pathogens. Both wide and narrow host range pathogens seem to be implicated in this, as well as other pest organisms (57, 102–109). However, once introduced into a new locality where these weeds did not previously occur and their fungal pathogens are absent, these plants may become invasive. Thus, bioinvasiveness can be regarded as a special case of the above-discussed Janzen-Connell hypothesis, i.e., the absence of natural enemies, including fungi.

SEED-BORNE PLANT PATHOGENS

Seed pathogens are important drivers of the soil-borne seed bank (110). Their importance is usually shown by measuring seed germination in fungicide-treated soils compared to untreated soils (111). An array of fungi have been found to be associated with decaying seeds, including typical seed-borne fungi that have infected the seed aboveground, such as *Alternaria* spp., and typical root pathogens such as *Neonectria* (syn. *Cylindrocarpon*) (112), as well as opportunists or secondary pathogens such as *Penicillium* spp. The effects of these organisms are strongly mediated by soil moisture content (113). Three ecological groups of organisms that reduce populations of soil-incubated seeds have been recognized: (i) primary pathogens able to kill the healthy seed under appropriate environmental conditions, (ii) opportunistic pathogens that contribute to seed decline after its vitality has been negatively affected by other environmental conditions, and (iii) pathogens that kill the seed directly after germination. Beckstead et al. (114) identified a negative correlation between the germination rate of seeds of *Bromus tectorum* and infection by the weak pathogen *Pyrenophora semeniperda*: thus, rapid germination leads to escape from the pathogen.

CONCLUSIONS

Fungal plant pathogens are ubiquitous and highly diverse. Together with other biotic stress factors they shape plant biodiversity in natural vegetation and in managed, species-rich grasslands, but in less biodiverse cropping systems they cause great losses. In these agroecosystems, resistance breeding has been only partially successful, and additional methods to manage pathogens are needed. The most important of these are measures to avoid soil-borne pathogens, the application of fungicides to control airborne pathogens, and production of clean seeds together with the application of seed disinfestation techniques. The basic prerequisites for an epidemic to develop are the same for natural and cropped ecosystems, i.e., a threshold density of host plants and pathogens, as well as proper environmental conditions. Host and pathogen density are the basic elements of the Janzen-Connell hypothesis and the dilution effect hypotheses, and thus, both hypotheses have much in common. Also, the phenomenon of overyielding of root biomass in plant mixtures relative to monocultures refers to these same basic elements of the disease triangle. The essential difference between natural and agricultural systems is that the replacement of diseased plants by other plant species occurs spontaneously in natural but not in agricultural systems. The introduction of fungal pathogens outside their natural distribution area is primarily due to international transportation of agricultural and horticultural products. This may have direct effects on existing or novel hosts and results in increased opportunities for hybridization or horizontal gene transfer, which can result in novel plant diseases. Although all plant pathogens occurring in agriculture originate from nature, it is questionable whether these pathogens have significant effects on natural ecosystems. Coevolution during domestication of crops may explain why the effects of these pathogens in natural environments seem limited.

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Key Ecological Roles for Zoosporic True Fungi in Aquatic Habitats

18

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INTRODUCTION

Phylogeny of Zoosporic True Fungi and Fungus-Like Microorganisms

The "Aquatic Phycomycetes" (*sensu* Sparrow) (1) constitutes an ecologically and economically important assemblage of eukaryotic microorganisms that share many morphological traits and ecological functions and interact with each other in the same aquatic ecosystems. There is molecular and structural evidence that the aquatic phycomycetes is a diverse, polyphyletic assemblage of species. For many years little research has been conducted with the aquatic phycomycetes, possibly because they were thought to be ecologically and commercially insignificant, but this perception has recently changed. Many of these species have been found to play key roles in biomass conversion in food webs (Fig. 1) and in the carbon cycle (2).

Baldauf (3, 4) divided eukaryotic organisms into eight supergroups based on a consensus phylogeny. The fungus-like species described in the "Aquatic Phycomycetes" *sensu* Sparrow (1) have been reassigned by Baldauf (3, 4) into four of these eight supergroups: the Opisthokonta, Straminipilia, Alveolata, and Rhizaria. The true fungi, the fungus-like organisms with uniflagellate zoospores (including chytrids, rumen chytrids, rosellids, and aphelids that are closely related to the true fungi), the mesomycetozoa (distantly related to the true fungi), the choanozoa, the slime molds, several groups of uniflagellate amoebae, and the phyla of multicellular animals were all placed in the Opisthokonta. One whiplash flagellum is usually present on zoospores of those species in the Opisthokonta, which produce motile stages for dispersal in their life cycles. Baldauf (3, 4) placed the fungus-like species with biflagellate zoospores into the supergroups Alveolata, Rhizaria, and Straminipilia. Many of the fungus-like species have heterokont zoospores with one anterior tinsel flagellum with mastigonemes and one posterior smooth whiplash flagellum without mastigonemes, but other species have zoospores with two whiplash flagella or one tinsel flagellum (5).

Ruggiero et al. (6) recently proposed a new higher-level classification of all living organisms. In this new proposal the eight supergroups of eukaryotes used by Baldauf (3, 4) have been replaced by five kingdoms: Animalia, Chromista, Fungi, Plantae, and Protozoa. The microorganisms within the "Aquatic Phycomycetes" *sensu* Sparrow (1) have been split by Ruggerio et al. (6) among three kingdoms: the kingdoms Chromista, Fungi, and Protozoa. The organisms within the supergroup Opisthokonta have been split among three kingdoms: the kingdoms Animalia, Fungi, and Protozoa. The microorganisms in the supergroups Amoebozoa, Discicristates, and Excavates have been placed into the kingdom Protozoa, and the organisms in the supergroups Alveolata, Rhizaria, and Straminipilia into the kingdom Chromista by Ruggerio et al. (6). Furthermore, some of the names for phyla have been changed by Ruggiero et al. (6). For example, the phylum Oomycota has been replaced by the phylum Pseudofungi.

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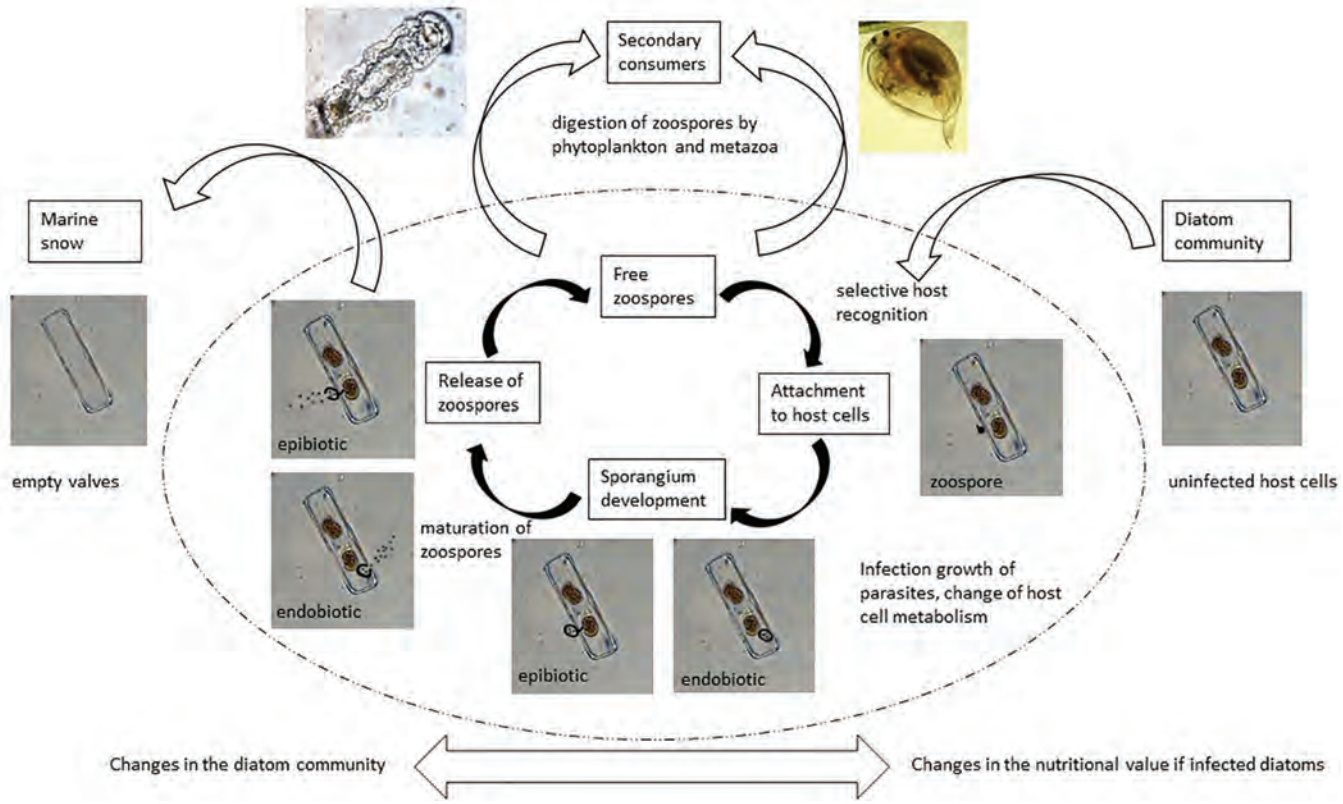


Figure 1 Schematic life cycle of endo- and epibiotic zoosporic parasites infecting marine diatoms. Besides the main cycle (solid black arrows), ecological effects on the marine planktonic and benthic community compositions, as well as interactions, are also depicted (unfilled outlined arrows).

Most fungus-like opisthokonts (Table 1) have a single, basal, whiplash, smooth flagellum on reproductive cells (zoospores and gametes), cell walls composed of chitin and plate-like mitochondrial cristae (1, 7–9). Most of the zoosporic true fungi, which Sparrow (1) and Barr (7) originally placed into the phylum Chytridiomycota (*sensu* Barr), have recently been reassigned into four newly described phyla (Blastocladiomycota, Chytridiomycota, Monoblepharidimycota, and Neocallimastigomycota) and the Olpidium clade (9–11). Sparrow (1) included *Rozella* in the phylum Chytridiomycota, but recently its species have been transferred to their own phylum, the Cryptomycota or Rozellida (12). Also, two new phyla of fungus-like microorganisms, which were unknown to Sparrow, have been recently added to the Opisthokonta supergroup: Aphelidea (9) and Mesomycetozoea (13). Busk et al. (14) reported about low sequence similarity between, and high overall diversity of, the genes encoding for secreted biomass-degrading enzymes produced by species of Blastocladiomycota, Chytridiomycota, Monoblepharidimycota, and Neocallimastigomycota.

Data from the sequences of rDNA and other genes have been used to describe a new phylum, the Rosallida (Cryptomycota), found in freshwater bog samples as a basal fungal clade (15) and the estimation of the molecular diversity of this clade as equivalent to that of the entire fungal kingdom (12). Species of Rosallida (Cryptomycota) are parasites of host species in the Chytridiomycota and Oomycota. Sparrow (1) described many species of *Rozella* based on their preferred hosts. The aphelids (phylum Aphelidea) are a small group of intracellular parasitoids of common species of eukaryotic phytoplankton with three known genera (*Aphelidium, Amoeboaphelidium*, and *Pseudaphelidium*) and 10 valid species that form, along with related environmental sequences, a very diversified group as well (16). The phylum Aphelidea is also new. The species in these two related phyla could easily be mistaken as chytrids because of similar morphology.

The use of software programs to compare DNA sequences of ribosomal and other conservative genes in living organisms has provided new insights into phylogeny and evolution. However, many questions have risen

Table 1 Currently described phyla in the supergroup Opisthokonta[a]

Phyla	Superphyla	Group	Cluster	Propagules
Basidiomycota	Holomycota	Eumycota	Dikarya	Thick-walled spores
Ascomycota				
Zygomycota			Sporangiospores	
			Zygospores	
Glomeromycota				
Olpidium clade[b]			Zoosporic	Zoospores
Monoblepharidomycota[b]			True fungi	(amoebae rarely)
Blastocladiomycota[b]				
Neocallimastigomycota[b]				
Chytridiomycota[b]				
Microsporidia		Opisthosporidia	True fungi	Walled spores
Rozellomycota[b]			Fungus-like	Zoospores
Aphelidea		Zoospores and amoebae		
Nucleariida				Amoebae
Fonticulida (MAFO)[c]				
Mesomycetozoea	Holozoa	Mesomycetozoea	Fungus-like	Thick-walled endospores
Cl. Dermocystida[b]				Zoospores
Cl. Ichthyophonida		Amoebae		
Acanthoecida		Choanozoa		Zoospores
Craspedia				
Fistasterea				
Animal phyla	Metazoa		Multicellular	

[a]Unifying characteristics are (i) uniflagellate zoospores usually, (ii) plate-like mitochondrial cristae; and (iii) if present, cell walls composed of chitin.
[b]Only the groups marked [b] were part of the "aquatic phycomycetes" as defined by Sparrow (1). The groups in bold fonts are zoosporic true fungi and will be considered in this article.
[c]MAFO, marine Fonticulida.

about phylogenetic relationships and evolutionary history from molecular studies, especially at the higher taxonomic levels, and remain unanswered. It is hoped that when complete genomic sequences of many more organisms become available, we will have a clearer understanding of phylogenetic relationships among all species of living organisms. The phylogenetic patterns proposed by Baldauf (3, 4), Adl et al. (17), and Ruggiero et al. (6) are based primarily on vertical gene transfer. Horizontal gene transfer has occurred between many species of both prokaryotes and eukaryotes, including fungi, and recent studies comparing complete genomes suggest that horizontal gene transfer has been more common than previously thought (18–20). Horizontal gene transfer must be considered in evolutionary history as well as vertical gene transfer. The precise phylogenetic relationships between many of the phyla of eukaryotic microorganisms are not clearly understood at present, and these higher-level classifications will probably be changed periodically in the near future as more sequence data become available.

In the past, lack of knowledge about aquatic zoosporic fungi and their ecological functioning has been compounded by misidentification of fungal zooflagellates, for example, as phagotrophic nanoflagellates (21). The identification of isolates of zoosporic true fungi and fungus-like microorganisms is complex, because of the lack of morphological characters visible in the light microscope, and often requires ultrastructural characterization of zoospores and other structures (22–24). Also chytrid sporangia and rhizoids exhibit phenotypic plasticity with morphological changes due to nutrient or other environmental conditions (25, 26). Furthermore, the parasitic species can be either epibiotic, with the zoosporangium on the surface and the rhizoids inside the host cell, or endobiotic, with both the zoosporangium and the rhizoids inside the host cell (27). Endobiotic parasites are very difficult to see under the light microscope. Recently, SSU rDNA sequences have been useful for understanding the phylogeny of zoosporic fungi (28).

Objectives of This Review

In this article, only the phyla that are closely related to the true fungi, which have adapted to aquatic environments and which produce motile zoospores usually with one whiplash flagellum, are considered (Table 1). All these species are within the supergroup Opistho-

konta (3, 4). Species of true fungi that produce thick-walled, nonmotile spores, but have adapted to aquatic environments, such as the aquatic hyphomycetes, are not considered in this review. Finally, the fungus-like organisms in the supergroups Alveolata, Rhizaria, and Straminipilia (or the kingdom Chromista) are not considered here either, because they are not considered to be true fungi, even though they share many morphological and physiological characteristics with true fungi and often inhabit similar environments. Some of these characteristics could have come across from species of true fungi to fungus-like species by lateral gene transfer during evolutionary history. For example, Richards et al. (19) and Richards and Talbot (20) discussed exchange of genes between groups of protists, in general, and specifically the lateral transfer of genes for pathogenicity from Ascomycota to Oomycota since these groups originally evolved. Although the Mesomycetozoea have some fungus-like characteristics, they are more closely related to the Choanozoa than the true fungi and will not be considered here.

This review focuses on some of the recent research on the ecology of zoosporic true fungal and fungus-like species in four phyla, Aphelidea, Chytridiomycota, Neocallimastigomycota, and Rosallida (Cryptomycota), and the interactions between these species and their substrates. Most of these species tend to be monocentric (unicellular), but there are polycentric (filamentous) species. Their lifestyles are considered based on the nature of symbiosis in aquatic habitats: saprotrophic, parasitic, and mutualistic lifestyles. It is essential for a thorough understanding of ecological processes that we correctly identify all species in all food webs in each ecosystem. Consideration of only species in the kingdom Fungi in ecosystems causes a dilemma, but our investigations have to begin somewhere, and thus, for this article, the scope of the topics covered must be limited.

DESTRUCTIVE MECHANISMS IN THE LIFE CYCLE OF ZOOSPORIC TRUE FUNGI

Zoospores

Zoosporic parasites produce motile zoospores. Some aspects of the physiology and ecology of zoospores have been reviewed by Fuller and Jaworski (29) and Gleason and Lilje (30). Motile zoospores are released from zoosporangia in large numbers into water and can swim for several hours. They are capable of navigating quickly through chemical gradients to their substrates (chemotaxis). After attachment, they encyst, germinate, and produce rhizoids, which penetrate their hosts and release digestive enzymes. The process of chemotaxis has only been studied in detail in the frog parasite *Batrachochytrium dendrobatidis* (31), in *Rhizophydium littoreum* (32), and in chytrid parasites of diatoms (33). In contrast, walled spores and cysts of other groups of parasites are carried by air or water currents to their hosts by chance or by vectors, such as animals.

Recent research into the chemotaxis of chytrid zoospores (33), using four marine chytrid-diatom tandem cultures in combination with different potential triggers, has shown that whole-cell extracts of the light-stressed hosts attracted the highest numbers of zoospores (86%), followed by the combined carbohydrate standard solution (76%), while all other compounds acted as weak triggers only. The triggers include aqueous host extracts grown under different stress conditions and standards of eight carbohydrates, six amino acids, five fatty acids, and three compounds known as compatible solutes (in individual and mixed solutions). In light of the results, it is tempting to hypothesize that pathogen zoospores were preferentially attracted to the fast-growing cells by photosynthesis-derived carbohydrate exudates. The chytrid-diatom tandem cultures comprised *Chytridium* sp./*Navicula* sp., *Rhizophydium* type I/*Nitzschia* sp., *Rhizophydium* type IIa/*Rhizosolenia* sp., and *Rhizophydium* type IIb/*Chaetoceros* sp., and could not be further isolated by the use of conventional methods (e.g., pollen) (33).

Rhizoids

The functions of rhizoids include penetration of the substrate, delivery of extracellular digestive enzymes, and absorption of food sources. Rhizoids exhibit phenotypic plasticity with morphological changes due to nutrient or other environmental conditions (19, 20).

Extracellular Enzymes

Proteases

Many species of zoosporic true fungi and fungus-like organisms can grow on substrates containing protein (1). Krarup et al. (34) demonstrated that some saprotrophic species of zoosporic true fungi also produce proteases. These extracellular enzymes are presumably released by rhizoids, which penetrate into utilizable nonliving substrates. Piotrowski et al. (35) found that the parasite *B. dendrobatidis* also produced proteases. Joneson et al. (36) describe significant lineage-specific expansions in three protease families (metallo, serine, and aspartyl proteases) in *B. dendrobatidis*. They show that expansion of these protease gene families occurred after the divergence of *B. dendrobatidis* from its an-

cestors. Finally, they demonstrated that the timing of these expansions predates the emergence of *B. dendrobatidis* as a globally important amphibian pathogen. Most likely, other groups of zoosporic parasites also use a variety of proteases in different families to digest the tissues in their animal hosts.

Cellulases

Rhizophlyctis rosea is a common chytrid, which grows quickly on plant debris containing cellulose in soil and aquatic ecosystems. In culture, this species is capable of the digestion of crystalline cellulose in the form of lens paper, filter paper, and powdered filter paper, and grows well with noncrystalline carboxymethyl cellulose or cellobiose, but cannot use starch or maltose as sole carbon sources in liquid and on solid media (37). Lange et al. (38) discovered that *R. rosea* zoospores excrete endocellulase GH45, which is thermostable.

The nature of cellulases excreted by other fungi is currently under study.

Cellulose, hemicellulose, and pectin are the polysaccharides in plant cell walls (39); cellulose has a linear structure of β-1,4-linked D-glucose that can be degraded to oligomers by the combined action of cellobiohydrolases, endocellulases, and lytic polysaccharide monooxygenases (LPMO) and further degraded to glucose by β-glucosidases (14). In higher plant species, cellulose is protected from degradation by the complex structures of plant cell walls including both covalent links between cellulose and hemicellulose and entanglement of the cellulose with other macromolecules. These structures make enzymatic digestion of cellulose difficult (14). Busk et al. (14) used PPR to classify GH families 1 to 131 and the AA families 9 to 11 (i.e., the LPMOs) into subfamilies. They found that enzymes with different properties are necessary for degradation of cellulose in different complex substrates. Also, that clustering of the fungi based on their predicted enzymes indicated that Ascomycota and Basidiomycota use the same enzymatic activities to degrade plant cell walls. They also found that the subfamilies could be used not only to predict the function of known GH-encoding genes but also to find all GH- and LPMO-encoding genes in a fungal genome and predict their functions (14).

GLOBAL DISTRIBUTION OF FRESHWATER AND MARINE TRUE FUNGI AND FUNGUS-LIKE MICROORGANISMS

Zoosporic true fungi (within the Eumycota and Opisthosporidia groups) (Table 1) are found in most aquatic and wet terrestrial ecosystems throughout the world. The phylum Chytridiomycota is one of the largest taxa of fungi (within the superphylum Holomycota) found in aquatic ecosystems (40). Since the 1960s, many species of freshwater zoosporic true fungi have been described (1, 7, 41, 42). However, ecological studies on these species have been limited. Phytoplankton and heterotrophic flagellates have traditionally been considered of primary importance in aquatic environments, while fungal diversity has been largely neglected (43). In recent years, discoveries in molecular methods of rDNA sequencing of small-subunit (SSU) rRNA genes (9, 44, 45), combined with broad meta-analysis of fungal populations in lacustrine, high-altitude soils under glaciers, and Arctic freshwater habitats (43, 46, 47), have revealed that zoosporic true fungi are predominant in these ecosystems and are likely to perform crucial ecological roles, like the degradation of pollen by saprotrophic freshwater zoosporic fungi (47).

Freshwater zoosporic fungi in freshwater ecosystems may be important parasites of phytoplankton and zooplankton. Planktonic chytrids are both parasitic and saprobic on the freshwater phytoplankton of Lake Inba, Japan (48). Chytridiomycota-like sequences with many novel lineages dominate the fungal diversity of temperate Lake Tahoe, United States, and freshwater Arctic habitats. Zoosporic fungi were positively correlated with either total chlorophyll a concentrations or with proportions of diatom sequences, indicating the likely parasitism of algae (43). *Olpidium gregarium* in the *Olpidium* clade was associated with declines in populations of rotifers (freshwater zooplankton) in the Rio Grande Reservoir, United States (49). In mesotrophic Lake Bourget in eastern France, zoosporic fungi in the *Rhizophydium* and *Nowakowskiella* clades were strongly represented among the fungi during May when the Chlorophyta and Bacillariophyta were also abundant (45). Zoosporic fungi may also be the primary decomposers of fine organic particulates and pollen grains in some freshwater environments (1). In the lakes of northeastern Germany, four fungal phyla were identified attached to pollen grains; 49% of the sequences were from the Chytridiomycota, predominantly the order Rhizophydiales. The zoosporic fungi in this study often appeared to be particle attached rather than free living (47). Pollen grains, leaves, insect and crustacean exoskeletons, pieces of snake skin, and live host species are excellent substrates for many chytrid species and have been used in the past to isolate chytrids into pure culture (1).

Historically, only a few species of zoosporic true fungi have been found in marine ecosystems, which led to the belief that these microorganisms were mainly lo-

cated in freshwater (50). Recent research has challenged this conclusion because of the discovery of many new marine species (discussed later).

SAPROTROPHS

The Effect of Environmental Factors on Growth and Development

Freshwater zoosporic true fungi encounter variations in physical conditions such as pH, soluble metals, temperature, and salinity. Although the temperature range of growth in aquatic zoosporic fungi has been little studied, many saprotrophic zoosporic fungi found in the soil have a maximum temperature for growth at 30°C, some species at 35°C and 37°C, and a few species at 40°C, but none survived incubation in liquid media above 45°C (51–53).

Fluctuations in pH are common in freshwater aquatic systems because of acid rainfall and runoff, sediments and fertilizers, algal blooms, and aquatic plant respiration. Zoosporic true fungi appear to tolerate extremely low pH, but not extremely high pH (1, 54). DNA sequences attributed to zoosporic fungi have been reported from the Rio Tinto River in Spain, which, as well as historically highly polluted with heavy metals, has been recorded as low as pH 2 (55). However, DNA sequences from the Rio Tinto River give no clues whether the inoculum was alive or dead; in addition, it cannot be excluded that the inoculum came from a tributary with higher pH. In comparison, zoospores from seven species of the plant parasitic *Phytophthora* (Oomycota), isolated from one reservoir, had optimal growth rates at acidic, neutral, or alkaline pH. However, some zoospores from all seven species survived at pH 3 and 11 (56).

Toxic metals affect many freshwater zoosporic fungi and fungal-like organisms with effects dependent on the type of metal and stage of the organism within its life cycle. Zoospores, which have a cell membrane rather than a cell wall, are more sensitive than any other stage. For example, gold is toxic to zoospores of the oomycete genus *Phytophthora* at 50 ppb (57). Some effects of metals on zoospores include increased encystment (58) and increased germination (59–61). A stimulatory effect on zoospore release at low levels of copper (10 ppm), lead (60 ppm), and zinc (10 ppm) was demonstrated for four isolates of zoosporic true fungi (62).

The level of osmolarity tolerated by zoosporic fungi varies based on their environment of origin. For example, the tolerance of an estuarine ecotype of *R. littoreum* to sodium, potassium, magnesium, and calcium ions was higher than freshwater ecotypes but lower than those in seawater (63), while maximum growth rate of the same ecotype in the presence of sodium ions was at 237 mM, approximately one-half the concentration of seawater (64). Zoosporic true fungi soil and freshwater isolates from several different genera were tested for growth at different salinities (65). All 20 isolates of zoosporic fungi in the orders Blastocladiales, Chytridiales, Cladochytriales, Rhizophydiales, Rhizophlyctidales, and Spizellomycetales grew on complex solid media supplemented with 170 mM NaCl, but not with 340 mM NaCl, indicating they would not survive in seawater.

Until recently, there have been relatively few studies on identifying marine zoosporic true fungi and fungus-like microorganisms, as opposed to freshwater zoosporic true fungi, which have been extensively studied (50). Those few studies mostly focused on chytrid parasites of dinoflagellates (50, 66) and diatoms (33). The identification of marine of zoosporic fungi has been problematic, because identification of species relied largely on morphology, which, in the case of some zoosporic fungi, with inconspicuous structural components, is extremely difficult (1, 67). However, the use of rDNA sequencing over the past few years has improved the species identification process, enabling researchers to quantitatively measure and compare the diversity and abundance of zoosporic fungi in marine environments, including the water column (9, 28, 44). Taylor and Cunliffe (68) recently used fungi-specific high-throughput sequencing and quantitative PCR analysis of plankton DNA samples collected over 6 years from the coastal site off Plymouth, United Kingdom, and assessed changes in the temporal variability of mycoplankton diversity (mainly Ascomycota, Basidiomycota, and Chytridiomycota) and abundance in relation to co-occurring environmental variables. Repeating mycoplankton blooms were linked to nitrogen availability and temperature, and specific relationships were found between mycoplankton and other plankton groups, for example, seasonal chytrid blooms matching diatom blooms in consecutive years. The study identified possible environmental drivers for mycoplankton diversity and abundance, with both diversity and abundance increasing with reduced salinity, and also when substrate availability was increased (68). With efficient and accurate methods of species identification now readily accessible to more researchers, we would expect that many more studies on the influence of environmental factors on diversity and distribution of marine zoosporic true fungi and fungus-like microorganisms will be undertaken in the near future.

Colonization Strategy

Saprotrophic zoosporic fungi colonize solid substrates in freshwater and soil ecosystems by using rhizoids, which release extracellular enzymes in order to penetrate the substrate to consume plant debris and fibers (1, 30, 69). (The colonization of fibrous plant material by fungi in the rumen is discussed in detail later in this review.) These enzymes are essential for the decomposition of organic matter in aquatic systems, because of their ability to degrade cellulose, the major component of vegetable debris (70, 71). Of interest is the recent discovery of an aquatic saprobe, *Synchytrium microbalum*, occurring within a genus of over 200 otherwise parasitic terrestrial species of zoosporic fungi (72).

PARASITES

Parasites of Marine Phytoplankton

Recent research revealed the presence of parasitic chytrids in marine coastal habitats. Several microalgae species, besides some dinoflagellates (66) and cyanobacteria (e.g., *Nodularia*), first and foremost bacillariophytes (diatoms), such as *Pseudo-nitzschia pungens* (73), *Chaetoceros*, *Amphora ovalis, Rhizosolenia, Biddulphia* (27, 74–76) (Fig. 2), *Thalassiosira, Skeletonema* (77), *Bellerochea*, and *Cylindrotheca* (78), were identified as host species for such zoosporic pathogens. Overall, only one chytrid, *Dinomyces arenysensis* infecting various dinoflagellates including toxic species of *Alexandrium*, has been identified and properly characterized by Lepelletier et al. (66, 79).

Traditionally, species description has been based on morphology of host and parasite (1, 67), which has been extended by discoveries in rDNA sequencing over the past years (9, 44). In addition, chytrids can show considerable morphological differences caused by nutrient availability or environmental conditions (25, 26). Also, many species might have been described previously, but their taxonomy has never been resolved or updated to fit our current phylogenetic concepts.

In general, molecular methods based on the amplification, cloning, and sequencing of SSU rRNA genes are a powerful tool to study the diversity of prokaryotic and eukaryotic microorganisms for which morphological features are not conspicuous (80). While DNA

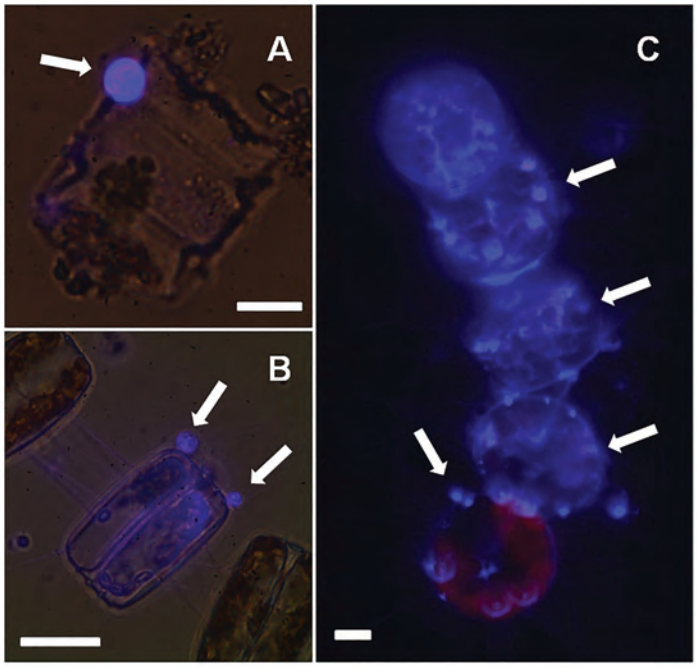


Figure 2 Representatives of the chytridiomycota infecting marine diatoms in phytoplankton net samples collected from the Skagaströnd area (northwest Iceland). *Odontella* (**A**) and *Thalassiosira* (**B**) single cell with multiple chytrid sporangia and colony with multiple infections (**C**). Pathogens were visualized by using calcofluor white stain in combination with transmission light and fluorescence excitation (UV light, 330 to 380 nm). Image by B. Scholz. Bar, 100 μm.

barcodes for terrestrial oomycetes are available and widely used (81), and genome regions for potential fungal barcodes have been identified (82), DNA barcodes for most other zoosporic parasites are, despite considerable effort (83), missing or not conclusive and the existing ones rarely allow identification, even at genus level (44). In all cases, further difficulties of DNA-based methods are primer bias within mixed samples going hand in hand with the troublesome establishment and maintenance of "pure" dual cultures of host and chytrid (44). However, current high-throughput sequencing approaches have revealed an unappreciated diversity and abundance of eukaryote parasites in the marine environment (de Vargas et al. [84]). Although reported as low in abundance and diversity (28, 84), marine true fungal sequences retrieved in metagenomic surveys are in most cases new to science (85). The phylum Chytridiomycota alone accounted for more than 60% of the reads in six near-shore sites around Europe (28, 86). Taking into account another fungal taxon likely to include parasites, such as the CRA (Cryptomycota/Rozell[ida]/Aphelid) group (28), this percentage increases by another 5%. Furthermore, the phylogenetic analysis of the same data set, together with other eukaryotic environmental sequences, suggested the existence of a whole clade of unknown marine chytrids and reported two clades branching close to known parasite species of phytoplankters (*Chytridium polisiphoniae* and *Amoeboaphelidium protococcarum*) (28).

In general, chytrid parasites may impact marine planktonic and microphytobenthic populations; therefore, large-scale, targeted approaches to monitor different habitats will result in increased knowledge about these parasites. In order to quantify chytrid infections in marine samples, the use of calcofluor white stain in combination with epifluorescence according to the protocol given by Rasconi et al. (87) for phytoplankton samples, as well as the much more cumbersome protocol for microphytobenthic samples (74), has shown the first acceptable results (27, 76). Another detection protocol involves the use of wheat germ agglutinin staining of chitin in environmental samples (28), with the side effect of staining Gram-positive bacteria due to its affinity for *N*-acetylglucosamine. The clear disadvantage of these methods is that they are time-consuming and do not lead to a secured identification of the parasite.

Although our knowledge of marine infectious disease dynamics is limited and often biased toward an economic point of view, there is much evidence to suggest that the occurrence of marine infectious diseases will increase with currently observed climatic trends (88), such as the alteration of epidemiology through effects on pathogen growth rates, transmission, virulence, and susceptibility, with potentially devastating results (79). Thus, it is necessary to draw attention to the importance of the host-parasite system in the marine environment, and emphasize the increasingly crucial role this system will play as, for example, aquaculture becomes more important in order to sustain the growing human population (79). Since infection by zoosporic parasites may not always significantly affect population size of hosts or microbial succession, in general, it is important to apply and develop new qualitative and quantitative techniques to study host-parasite interactions.

Parasites of Freshwater Phytoplankton

The infection of freshwater phytoplankton by chytrid parasites has been documented for a variety of dinoflagellates, diatoms (Fig. 3), and cyanobacteria and in a variety of environments. There is accumulating evidence that the pelagic zone of lakes is housing a significant unexplored diversity in chytrid species (89). These discoveries are particularly interesting when the system concerned is exhibiting significant ecological and environmental change. For example, Leshem et al. (90) recently characterized a new species of chytrid parasit-



Figure 3 Chytrid parasites infecting a freshwater diatom (*Pinnularia* sp.) collected from a freshwater pond in Centennial Park, Sydney, Australia. Image by D.J. Macarthur. Bar, 50 μm.

izing the dinoflagellate, *Peridinium gatunense*, in Lake Kinneret, Israel, a system that has been the subject of studies detailing its pronounced shifts in phytoplankton community structure (91). Similarly, chytrid infection of the cyanobacteria *Planktothrix* has been extensively analyzed in Lake Kolbotnvannet, Norway, a system that has experienced increased urbanization over the past 35 years (92). In this study, it was found that improved water quality conditions, due to better water quality management practices, favored a single *Planktothrix* chemotype. It was hypothesized that the loss of diversity would allow the chytrid species known to parasitize *Planktothrix* to dominate and therefore reduce the dominance of the single chemotype and return the system to a diverse equilibrium. Contrary to the hypothesis, it was found that the chemotype-chytrid relationship remained in equilibrium. It was therefore further hypothesized that this was due to the *Planktothrix* moving deeper in the water column, taking advantage of increased light penetration as a result of the improved water quality. As a result of this movement, environmental stresses, such as decreased temperature, have increased for the chytrids and limited their ability to dominate. These contextual studies are beginning to add a further dimension into our perspective on the anthropogenic effects of ecological interactions. Elucidated host-parasite relationships are emerging as excellent models for garnering insight into the traditionally opaque ecological dynamics of aquatic food webs (93). This insight has been experimentally confirmed and is known informally as the "Mycoloop" (94).

The Mycoloop is a trophic transfer loop, where the release of zoospores by chytrid parasites of phytoplankton provides an additional and potentially very important source of food for grazer zooplankton (94). This dynamic is particularly crucial where phytoplankton species are not a suitable food source for zooplankton, such as the large diatom *Asterionella* (95). The significance of the presence of chytrid zoospores in food webs has been explored mathematically by Grami et al. (96), who did extensive sampling of Lake Pavin in France and quantified bacteria, heterotrophic nanoflagellates, nanoplankton and microphytoplankton, ciliates, metazooplankton, and chytrids. These data combined with physiochemical measurements of water were used to construct a pelagic food web model. The inclusion of chytrids in this model resulted in significant increases in lake carbon flows, suggesting a mathematical replication of the conceptual Mycoloop. In addition, the presence of chytrids in the system increased the number of trophic links and the length of trophic pathways, which contribute to the overall stability of the system (2).

Much research has been committed to attempting to explain and manage the potentially devastating toxins produced by cyanobacteria, particularly in light of global elevated temperatures and stratification in water bodies favoring the physiological strategies employed by these prokaryotes (97, 98). Studying the relationship between the filamentous toxin-producing cyanobacteria *Planktothrix* and its chytrid parasite *Rhizophydium megarrhizum* revealed that the synthesis of microcystins in *Planktothrix* provided a significant defensive aid against infection, whereas mutants of *Planktothrix* with toxin-synthesis genes knocked out exhibited an enhanced vulnerability to parasitic infection (99). This study is arguably the most concrete in discerning the ecological function of toxins synthesized by phytoplankton, an area that is still characterized by ambiguity.

Evidence is emerging that the interactions between the chytrid parasite and its host may involve strong phenotypic selection pressures. An unpublished study by D. J. Macarthur (NSW Environmental Trust 2006/RDS/0007) involved the inoculation of cultures of the bloom-forming cyanobacteria *Anabaena circinalis* and *Microcystis aeruginosa* with diatoms infected with chytrids and with pure cultures of chytrids in the Rhizophydiales. One of these chytrids, which was isolated in pure culture from a phytoplankton sample containing infected diatoms (by E. Lefèvre, Department of Civil and Environmental Engineering, Duke University, Durham, NC) and was tentatively identified as *Rhizophydium* sp., adapted to the new host environments by parasitizing both species of cyanobacteria. These results support the theory of Kagami et al. (100) that the host range of chytrid parasites, often thought to be host-specific, could be altered by environmental stress. More interesting, however, was an apparent enhancement of growth and survival of *A. circinalis* cultures (101). Apparently, there were benefits to both the chytrid parasite and the cyanobacteria host. However, more data are required to unravel the complicated interactions between the chytrid parasite and its host. The growth and survival of chytrids are quite sensitive to physical factors, such as moisture, temperature, salinity and dissolved oxygen (102), and possibly toxic chemicals. With global warming and increased environmental deterioration, more research is required on the ecology of the chytrids in aquatic ecosystems. There is growing evidence that the survival of many aquatic ecosystems may depend on the activities of chytrids along with other groups of microorganisms.

More recently, Frenken et al. (103) demonstrated that warming can significantly interact with chytrid-

bloom dynamics. Mesocosms with a blooming population of the diatom *Asterionella*, which was also being parasitized by chytrids resembling *Zygorhizidium planktonicum*, exhibited earlier bloom termination times in response to a warming treatment of 4°C. This result was accompanied by cascade effects in the zooplankton population; *Keratella* numbers peaked earlier and at a higher density, but exhibited a more rapid and dramatic decline in the postbloom stages of the mesocosms. These results hint at the potentially huge significance chytrid parasites may have in aquatic ecosystems.

A key but contentious theory in evolutionary ecology, the Red Queen Hypothesis (104) has also been explored in the context of chytrid parasites of freshwater phytoplankton. The theory states that host-parasite relationships are evolutionary balancing acts, where hosts and parasites are coevolving to resist infection, or infect at a higher incidence, respectively. The theory is conceptually pleasing as a driver of rapid microbial evolution, but Gokhale et al. (105) found that a mathematical representation of the theory, based on classic Lotka-Volterra dynamics, resulted in frequent system collapse rather than a stable persisting relationship. However, a field study examining the long-term relationship between a genetically monotonous population of *Planktothrix* and their chytrid parasite in sediment cores from Lake Kolbotnvannet, Norway, showed a very stable relationship over many years (92). As discussed previously, there are several plausible reasons for this, most notably the seasonal patterns of the lake providing intermittent "thermal refugia," where the temperature drops below the tolerable threshold for the chytrid, but not for *Planktothrix*, allowing the host population to stabilize. The presence of hyperparasites, such as the zoosporic *Rozella* sp., in the system could also theoretically act as an additional pressure on the parasite population (106), but the influence of these enigmatic organisms is still very poorly characterized.

Similar to the paradigm of negativity surrounding blooms of phytoplankton, which may be perfectly natural and even essential biological "resetting" events, there is a negativity associated with diseases and parasitism, mostly derived from their potentially devastating economic effects. This dynamic has been associated with chytrid infection of commercial algae crops (107), which, because of rigorous strain selection, suffer substantially from infection. However, being a component of the so-called biological "dark matter" (108), these fungi should be considered as evidence of further uncharted levels of biodiversity, which deserves a certain respect and appreciation.

SYMBIONTS

Distribution

Fungi that thrived in the absence of oxygen were first detected in the form of motile zoospores in the rumen fluid of ruminant animals by Liebetanz and Braune as early as 1910 (109, 110). At the time, these remarkable organisms were mistakenly described as ciliated protozoa. More than 6 decades later, Orpin consistently observed vegetative fungal growth while attempting to isolate ciliated protozoa from the sheep rumen (111, 112). The morphology of these microorganisms and the presence of chitin in their cell walls provided further proof that they were in fact spores of a new lineage of true fungi (111, 113, 114). Since their original discovery in sheep, anaerobic gut fungi have been isolated from and detected based on microscopy in the rumen, hindgut, and feces of at least 24 different host genera representing eight different animal families (115, 116). Among these are all foregut fermenters, including the ruminants (families Bovidae and Cervidae), pseudoruminants (e.g., hippopotamus, camel, llama, alpaca, and vicugna) and foregut nonruminants (e.g., marsupials), as well as some hindgut fermenters (e.g., elephant, horse, and rhinoceros). Anaerobic fungi have further been identified in the intestine of laboratory mice (117), in guinea pigs (118), and in some larger herbivorous rodents, e.g., the mara (*Dolichotis patagonum*) (119), but not in some other small hindgut-fermenting animals (presumably because of the shorter duration time of ingested plant material in their smaller cecum) (116). The panda, which lacks foregut and hindgut fermentation chambers, has also not been found to harbor anaerobic fungi (120), and this has been attributed to the simplicity of its alimentary tract (116). Anaerobic fungi were, however, detected in the gut of the marine iguana (*Amblyrhynchus cristatus*) (121, 122). These herbivorous reptiles feed on marine algae and have fermentative digestion processes similar to those in herbivorous mammals. However, some other herbivorous reptiles (i.e., tortoises) do not seem to be colonized by anaerobic fungi (122). In the sea urchin, *Echinocardium cordatum*, zoosporic true fungi have been observed to make up part of the microflora in the anterior cecum, intestinal cecum, and coelomic fluid (123). Some parts of the digestive system are thought to be relatively anoxic and the microflora to be obligately anaerobic. This species of sea urchin digs and feeds in anaerobic muddy substrates in marine environments. Sea urchins are herbivores and feed primarily on marine macroalgae. Whether the zoosporic true fungi observed in *E. cordatum* are phylogenetically related to rumen fungi

found in herbivorous mammals and reptiles is not yet known because of the lack of molecular data.

Life Cycle and Ecology

The strictly anaerobic fungi (phylum Neocallimastigomycota, order Neocallimastigales) (124) play a pivotal role in the functioning of the alimentary tract of herbivorous mammals and reptiles (125), and so it is not surprising that anaerobic fungi comprise some 20% of the microbial biomass of sheep feeding on high-forage diets (126). Forages mainly consist of crystalline cellulose and noncrystalline hemicellulose from plant cell walls and represent the most important carbon and energy sources for terrestrial herbivorous mammals and reptiles. In a mutualistic relationship with their host, anaerobic fungi, together with bacteria, archaea, and protozoa, realize the degradation of the ingested plant material, which the host could otherwise not use (114, 125). While sexual reproduction has not been reported for anaerobic fungi of the phylum Neocallimastigomycota, asexual reproduction occurs through the production of flagellated zoospores from sporangia (127). The formation of sporangia, zoospore differentiation, and their subsequent maturation are thought to be induced by heme and other related porphyrins, which are released from ingested plant material (111, 112, 118, 128, 129). Two types of zoospores have been observed: monoflagellate and polyflagellate zoospores. During this motile stage, anaerobic fungi rapidly locate surfaces for colonization *via* chemotaxis toward soluble sugars that leak from damaged plant material (111, 112, 130). Upon attachment to the fiber, the zoospores shed their flagella and form a cyst (111, 114). Development of the cyst varies depending on whether the fungus is monocentric (one sporangium develops from single zoospore) or polycentric (many sporangia develop from a single zoospore) (131). In monocentric taxa, the nucleus remains within the cyst (endogenous), which enlarges to form a zoosporangium. Thus, the rhizoids remain anucleate. In polycentric taxa, the nuclei migrate into the rhizoidal system (exogenous), thereby enabling the formation of multiple sporangia on each thallus (125, 129). The genera *Caecomyces* and *Cyllamyces* form bulbous holdfasts and appear to represent intermediate forms (132). In both genera, nuclei are observed in the holdfast, consistent with exogenous development, and, in the case of *Cyllamyces*, also in the branched sporangiophores. However, the development of these thalli, while not as strictly determinate as the monocentric/rhizoidal *Neocallimastix* and *Piromyces*, is clearly more limited than in the polycentric/rhizoidal *Anaeromyces* and *Orpinomyces* (116). The rhizomycelium of the anaerobic fungi is characterized as being either filamentous or bulbous, the latter possessing spherical holdfasts (132, 133). Distinct intercalary rhizoidal swellings are occasionally observed in the genus *Oontomyces* (134). The developing rhizoids physically penetrate rigid, undamaged plant cell walls using an appressorium-like structure (113, 135–138). The so revealed internal plant tissues become accessible to enzymatic breakdown and provide nutrients that enable the development and maturation of the sporangia, which may then produce and release zoospores before the cycle repeats (139, 140). The initial attack of plant fiber by anaerobic fungi appears to facilitate a more rapid breakdown of forage feed by fibrolytic bacteria, which have difficulty penetrating large food particles (141, 142). In addition to using physical force, anaerobic fungi also produce cellulolytic and hemi-cellulolytic enzymes, in contrast to their mammalian and reptilian hosts that mediate the breakdown of plant cell wall carbohydrates, especially lignocellulose (143–147), thereby supplying reducing equivalents in the form of hydrogen to the bacterial and archaeal communities (148–150). The presence of hydrogen- and ATP-generating hydrogenosomes in anaerobic fungi was first demonstrated in the rumen anaerobic fungus *Neocallimastix patriciarum* (151). Axenic cultures of fungal species within this genus produced hydrogen during a (bacterial-type) mixed-acid fermentation of carbohydrates (152, 153), but the mechanism of hydrogen production had not been established and the presence of specialized redox organelles not reported. Yarlett et al. (151) showed hydrogenase activity in both the motile zoospore stage and the nonmotile vegetative reproductive stage of the fungus. Since then, the mitochondrial origin of anaerobic fungal hydrogenosomes has been demonstrated (154, 155), and their presence confirmed in several other genera of anaerobic fungi (143, 156). The end products of microbial fermentation, including short-chain fatty acids such as acetate, propionate, and butyrate, are taken up by the host via the epithelium (148–150). Anaerobic fungi successfully disperse between hosts, and it is believed that transition occurs via the formation of aerotolerant life stages, such as the two- to four-chambered spores formed by some *Anaeromyces* spp. (132, 157), although the processes whereby these develop and later germinate remain to be elucidated (116).

Taxonomy

Currently, the anaerobic fungi are classified in a single order (Neocallimastigales) within the recently erected phylum Neocallimastigomycota (124) and are most closely related to the Chytridiomycota. Within this

order, eight genera of anaerobic fungi are recognized: *Anaeromyces* (158), *Buwchfawromyces* (159), *Caecomyces* (160), *Cyllamyces* (132), *Neocallimastix* (161), *Oontomyces* (134), *Orpinomyces* (162), and *Piromyces* (160). Since 18S rRNA genes of anaerobic fungi are too conserved to distinguish between different genera and species, the internal transcribed spacer 1 (ITS1) has been recommended as molecular barcode marker (82, 157, 163). Several studies used this gene region to evaluate anaerobic fungal diversity in the alimentary tracts of a wide range of wild and domesticated, ruminant and nonruminant herbivores (122, 164–172). Some of these studies reported the existence of several as yet uncultivated novel groups of anaerobic fungi (122, 168, 169). Based on anaerobic fungal ITS1 sequence and secondary structure information, Koetschan et al. (173) established a comprehensive and stable phylogeny and pragmatic taxonomy of the Neocallimastigomycota, which may be used for highly resolved taxonomic assignment of high-throughput sequence data. Studies using next-generation sequencing technologies have recently allowed comprehensive new insights into the diversity and community structure of anaerobic fungi in the guts of a wide range of herbivorous animals (122) or in the rumens of cattle (171, 174), deer (175), or sheep feeding on different diets (176) or exhibiting different methane emission phenotypes (177). It appears that even hosts from highly distant families that feed on rather unique diets can harbor large numbers of shared sequence types (122), and that there are no (apparent) geographical restrictions in the occurrence of anaerobic fungal sequence types (168). It is likely, however, that some anaerobic fungal species are indeed host specific, as has been suggested for some of the novel as-yet uncultivated clades (122) and the recently described species *Oontomyces anksri*, which was isolated from the Indian camel (134). The recently conducted sequence-based surveys have broadened our understanding of the ecology of anaerobic fungi and provided a glimpse into their global genus-level diversity. Interestingly, the relative lack of overlap between the novel lineages identified in the different studies suggests that additional, yet-unknown novel candidate genera may exist in nature (116).

CONCLUSIONS

The diversity and abundance of zoosporic true fungi have been recently analyzed by using fungal sequence libraries and advances in molecular methods, such as high-throughput sequencing. This review focuses on four phyla, the Aphelidea, Chytridiomycota, Neocallimastigomycota, and Rosellida (Cryptomycota). Zoosporic fungi appear to be both abundant and diverse in many aquatic habitats around the world, with abundance exceeding other fungal phyla and numerous novel genetic sequences identified. Zoosporic fungi are able to survive extreme conditions, such as high and extremely low pH, but more work remains to be done. They appear to have an important role as saprobes in the decomposition of particulate organic substrates such as pollen, plant litter, and dead animals; as parasites of zooplankton and algae; as parasites of vertebrate animals (such as frogs); and as symbionts in the digestive tracts of mammals. Some chytrids cause economically important disease of plants and animals. They also regulate sizes of phytoplankton populations. Further metagenomics surveys of aquatic ecosystems are expected to enlarge our knowledge of the diversity of the zoosporic true fungi. Coupled with studies on their functional ecology, we are moving closer to unraveling the role of zoosporic fungi in carbon cycling and the impact of climate change on zoosporic fungal populations.

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How Fungi Sense Their Environment

IV

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Nutrient Sensing at the Plasma Membrane of Fungal Cells

19

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INTRODUCTION

To respond to the changing environment, cells must be able to sense external conditions. This is important for many processes including growth, mating, the expression of virulence factors, and several other regulatory effects. Nutrient sensing at the plasma membrane is mediated by different classes of membrane proteins that activate downstream signaling pathways: nontransporting receptors, transceptors, classical and nonclassical G-protein-coupled receptors, and the newly defined extracellular mucin receptors. Nontransporting receptors have the same structure as transport proteins, but have lost the capacity to transport while gaining a receptor function. Transceptors are transporters that also function as a receptor, because they can rapidly activate downstream signaling pathways. In this review, we focus on these four types of fungal membrane proteins. We mainly discuss the sensing mechanisms relating to sugars, ammonium, and amino acids. Mechanisms for other nutrients, such as phosphate and sulfate, are discussed briefly. Because the model yeast *Saccharomyces cerevisiae* has been the most studied, especially regarding these nutrient-sensing systems, each subsection will commence with what is known in this species.

NONTRANSPORTING RECEPTORS SENSING GLUCOSE

Glucose Sensing by Snf3 and Rgt2 in *S. cerevisiae*

The uptake of glucose, the preferred carbon source of *S. cerevisiae*, is mediated by 17 closely related hexose transporters (1). Their expression is coordinated according to the availability of extracellular glucose, which is sensed by the two glucose receptors, Snf3 and Rgt2 (2) (see "GPCR-mediated carbon sensing in *S. cerevisiae*" and Fig. 1). Snf3 and Rgt2 sense the internal/external ratio of glucose to adjust glucose uptake (3), which is also influenced by intracellular glucose metabolism (glycolysis) (4). Although these receptors are structurally similar to hexose transporters, they cannot transport glucose and contain unusual long C-terminal tails that are important for glucose sensing (5).

In the absence of glucose, the Rgt1 transcriptional repressor is found in the nucleus, interacting with the corepressors Mth1-Std1 (6), thereby recruiting the general corepressor complex Ssn6-Tup1 to the promoters of the different *HXT* genes, blocking their transcription (7). Snf1-dependent phosphorylation of Rgt1 triggers its repressor activity as well as its propensity to bind DNA (8). In addition, Snf1 also affects *HXT* expression via repression of the carbon catabolite repressors, Mig1/2, which in turn repress the expression of *STD1*, *MTH1*, and *SNF3* genes (9). Glucose detection by Snf3-Rgt2 recruits the corepressors Mth1-Std1 to the plasma membrane where they are subsequently phosphorylated by type I casein kinases Yck1/2 (10). Upon phosphorylation, Mth1-Std1 are targeted for SCF E3 ligase Grr1-mediated ubiquitination and proteasomal degradation (11). Collectively, these events lead to a protein kinase A (PKA) dependent hyperphosphorylation of Rgt1, which induces the dissociation of Ssn6-Tup1 from the DNA, resulting in the derepression of the *HXT* genes (12, 13).

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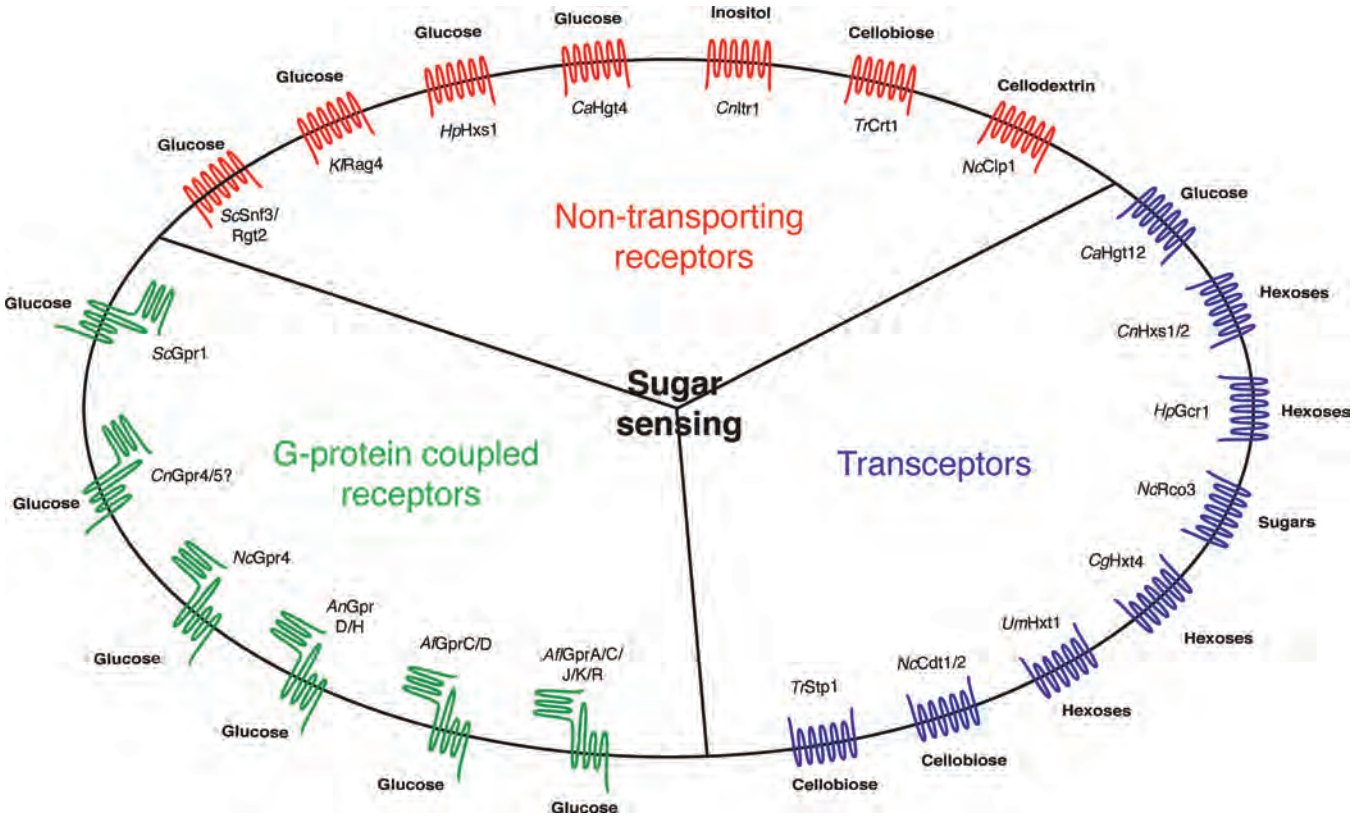


Figure 1 Sugar-sensing proteins in the plasma membrane of fungal cells. Nutrient-sensing proteins in general can be divided into nontransporting receptors, transceptors, and G-protein-coupled receptors (GPCRs). Nontransporting sugar receptors include the *Saccharomyces cerevisiae* (*Sc*) glucose sensors Snf3/Rgt2, the *Kluyveromyces lactis* (*Kl*) glucose sensor Rag4, the *Hansenula polymorpha* (*Hp*) glucose sensor Hxs1, the *Candida albicans* (*Ca*) glucose sensor Hgt4, the *Cryptococcus neoformans* (*Cn*) inositol sensor Itr1, the *Trichoderma reesei* (*Tr*) cellobiose sensor Crt1, and the *Cn* cellodextrin sensor Clp1. Sugar transceptors include the *Ca* glucose sensor Hgt12, the *Cn* hexose sensors Hxs1/2, the *Hp* hexose sensor Gcr1, the *Neurospora crassa* (*Nc*) sugar sensor Rco3, the *Colletotrichum graminicola* (*Cg*) hexose sensor Hxt4, the *Ustilago maydis* (*Um*) hexose sensor Hxt1, the *Nc* cellobiose sensors Cdt1/2, and the *Trichoderma reesei* (*Tc*) cellobiose sensor Stp1. Sugar GPCRs include the *Sc* glucose sensor Gpr1, the *Cn* glucose sensors Gpr4/5, the *Nc* glucose sensor Gpr4, the *Aspergillus nidulans* (*An*) glucose sensors GprD/H, the *Aspergillus fumigatus* (*Af*) glucose sensors GprC/D, and the *Aspergillus flavus* (*Af*) glucose sensors GprA/C/J/K/R.

Homologs of the *Sc*Snf3-*Sc*Rgt2 pathway have been identified in other yeasts (Fig. 1). In *Kluyveromyces lactis*, this is the Rag4 sensor (14), which regulates the expression of the glucose transporter gene *RAG1* (15). In addition, the downstream regulatory pathway is similar to that described for *S. cerevisiae* (16–18). In the methylotrophic yeast *Hansenula polymorpha*, the *Hp*Hxs1 is nonfunctional as a glucose transporter, but is responsible for the glucose-dependent induction of the functional transporter *Hp*Hxt1 (19). The C terminus of this receptor is essential for its function, and the mutation of one conserved residue (R204K) converts it into a constitutively signaling form.

Carbon Sensing in *Candida albicans* and *Cryptococcus neoformans*

C. albicans has over 20 hexose transporter homologs (20). Among them, Hgt4, an ortholog of the *S. cerevisiae* glucose receptors Snf3 and Rgt2, has been identified as a high-affinity glucose sensor (21) (Fig. 1). Hgt4 shares sequence and structure similarity with other hexose transporters, with the exception of a long C-terminal tail containing 254 amino acids, similar to Snf3 and Rgt2. Hgt4 is required for glucose induction of the hexose transporter genes *HGT12*, *HXT10*, and *HGT7*. Mutagenesis demonstrated that the *hgt4* mutant is defective in growth on fermentable sugars, such as fruc-

tose, mannose, and glucose. The *hgt4* mutants are hypofilamentous, indicating that Hgt4 affects the yeast-to-hyphal morphological switch. Interestingly, Hgt4 is also required for fungal virulence because the mutant showed attenuated virulence in a murine model for systemic infection. These findings demonstrate that *C. albicans* cells need to sense and regulate sugar levels for fungal filamentation during infection. The downstream regulatory mechanism of glucose repression in *C. albicans* is conserved with its counterpart in *S. cerevisiae* (12, 22). However, it remains unclear whether Hgt4 has glucose uptake activity or not.

Also in *C. neoformans*, glucose sensing and utilization are critical for its development and virulence. Besides being utilized as a preferred energy source for cell growth, glucose is required for capsule production both as a substrate and a signaling molecule. Glucose induces capsule enlargement through the Gpa1-cAMP-PKA signal transduction pathway, which plays a central role in fungal virulence (see "GPCR-mediated carbon sensing in *C. albicans* and *C. neoformans*" below) (23). Despite its biological significance, glucose sensing is less well understood in *C. neoformans*, in comparison with *S. cerevisiae* and *C. albicans*. There are ~50 transporters that share high sequence similarity with hexose transporters (24). Although some of them may transport sugars other than glucose, how this large family of hexose transporters is regulated remains unknown. Among these hexose transporter homologs, Hxs1 and Hxs2 share the highest sequence identity with *Sc*Rgt2/*Sc*Snf3 glucose receptors, but neither of them has a long C-terminal tail (24). Hence, it is possible that the *C. neoformans* glucose-sensing system is different from those of *S. cerevisiae* and *C. albicans*. These two proteins will be discussed in the section "Transceptor-Mediated Carbon Sensing in *C. albicans* and *C. neoformans*" because evidence suggests that they may have both transport and regulatory functions (Fig. 1).

Inositol is a small carbohydrate molecule that functions as an essential structural and signaling molecule in eukaryotes, including fungi (25). In *C. neoformans*, inositol can be used as a sole carbon source (26). Inositol can also stimulate *Cryptococcus* mating (27). As one of the most abundant metabolites in the mammalian brain (28), inositol utilization is required for *C. neoformans* virulence in murine infection models by promoting brain infection (29–32). Therefore, *C. neoformans* is likely to utilize the abundant inositol available inside the brain for its pathogenesis. Inositol can also stimulate *C. neoformans* capsule growth, which may contribute to its role in fungal virulence. The cryptococcal genome reflects the evolutionary adaptations associated with the expanded role of inositol in this organism. In particular, *C. neoformans* contains an unusually large number of inositol transporters (*ITR*s) that consist of more than 10 members (27, 29). Functional analysis of the *ITR* gene family demonstrated that two members (Itr1a and Itr3c) have high inositol uptake activity, while the function of the other members remains undefined. Among them, Itr1 was suggested as an inositol transceptor because it does not show uptake activity in the yeast heterologous system, it regulates the expression of *ITR* genes, and the *itr1* single mutant exhibits a defect in mating, hyphal production, and sporulation (29, 32). Therefore, transceptors for inositol may exist in *C. neoformans*.

Carbon Sensing in Filamentous Fungi

The mechanisms by which filamentous fungi sense saccharides in the environment are beginning to emerge. It is becoming increasingly clear that, because of the metabolic plasticity of filamentous fungi and their ability to utilize numerous nutrient sources, sensory mechanisms and pathways are far more complex than those in yeasts. This knowledge has been fundamental to the industrial application of filamentous fungi in the production of second-generation biofuels and green chemistries. Lignocellulolytic filamentous fungi, such as *Trichoderma reesei, Aspergillus nidulans*, and *Neurospora crassa*, secrete an array of carbohydrate-active enzymes (CAZymes) that deconstruct and utilize lignocellulose. Primarily, the activity of a few scavenging fungal enzymes releases potent saccharide inducers, such as cellobiose, from the plant biomass, which induces the secretion of hundreds of CAZymes by the fungus, promoting lignocellulose deconstruction (33). However, the detection of simple saccharides, such as glucose, xylose, or an abundance of cellobiose, results in CreA/Cre1-mediated carbon catabolite repression (33). Therefore, distinct mechanisms for sensing plant-derived saccharides exist to enable the efficient regulation of fungal metabolism and alternative carbon usage.

In contrast to the aforementioned yeasts, homologs of the Snf3 and Rgt2 glucose receptors have not been functionally defined in filamentous fungi. In *T. reesei* a putative major facilitator superfamily (MFS) sugar transporter Crt1 was required for full cellulase activity and growth on cellulose or lactose (34). The absence of Crt1 did not compromise cellobiose or sophorose uptake, implying that the function of Crt1 in cellulase induction did not rely on its transporting activity. However, in *N. crassa* cellobiose promotes cellulase

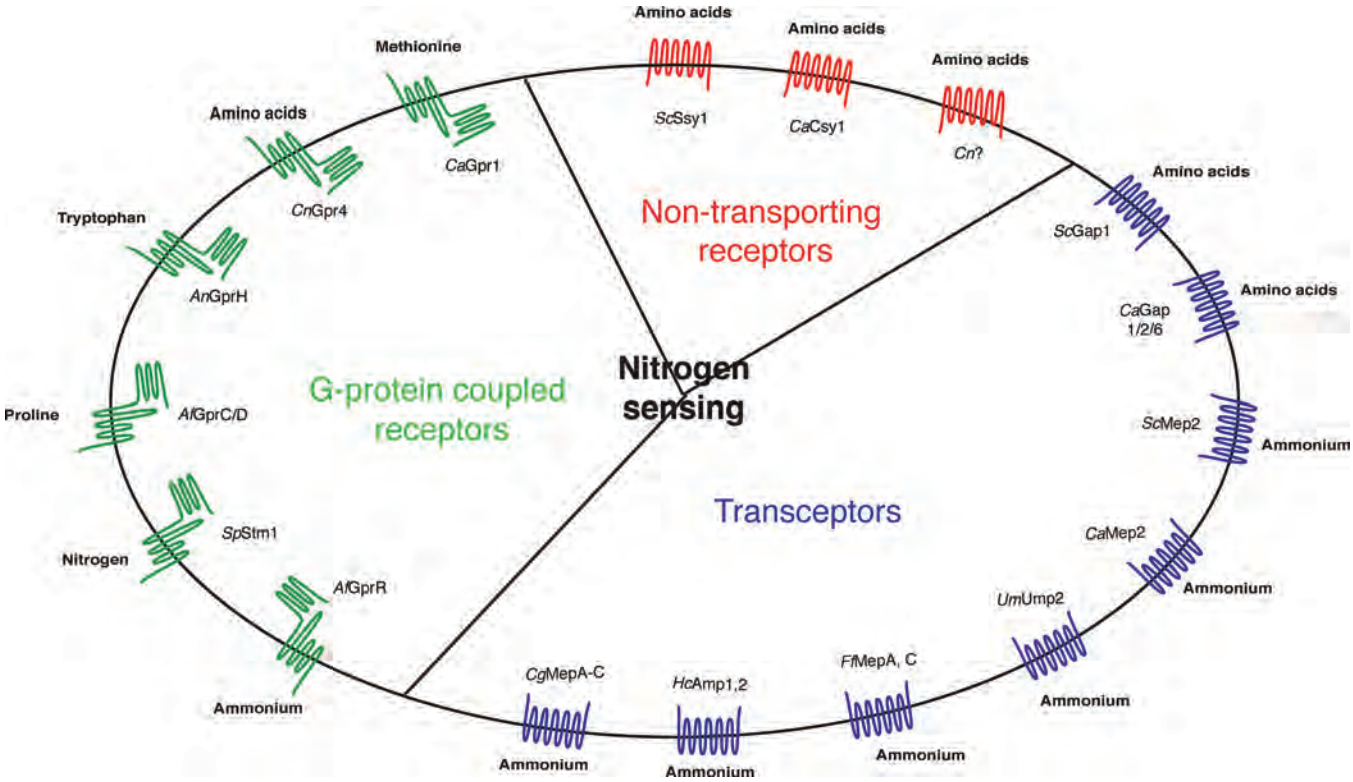


Figure 2 Nitrogen-sensing proteins in the plasma membrane of fungal cells. Nutrient-sensing proteins, in general, can be divided into nontransporting receptors, transceptors, and G-protein-coupled receptors (GPCRs). Nontransporting nitrogen receptors include the *Saccharomyces cerevisiae* (*Sc*) amino acid sensor Ssy1, the *Candida albicans* (*Ca*) amino acid sensor Csy1, and an unknown *Cryptococcus neoformans* (*Cn*) amino acid sensor. Nitrogen transceptors include the *Sc* amino acid sensor Gap1, the *Ca* amino acid sensors Gap1/2/6, the *Sc* ammonium sensor Mep2, the *Ca* ammonium sensor Mep2, the *Ustilago maydis* (*Um*) ammonium sensor Ump2, the *Fusarium fujikuroi* (*Ff*) ammonium sensors MepA-C, the *Hebeloma cylindrosporum* (*Hc*) ammonium sensors Amt1-3, the *Tuber borchii* (*Tb*) ammonium sensor Amt1, and the *Colletotrichum gloeosporioides* (*Cg*) ammonium sensors MepA-C. Nitrogen GPCRs include the *Ca* methionine sensor Gpr1, the *Cn* amino acid sensor Gpr4, the *Aspergillus nidulans* (*An*) tryptophan sensor GprH, the *Aspergillus flavus* (*Af*) proline sensor GprC/D, the *Schyzosaccharomyces pombe* (*Sp*) nitrogen sensor Stm1, and the *Af* ammonium sensor GprR.

production independent of glucosidase activity, indicating that cellobiose itself is the inducer for the cellulolytic regulon (35), suggesting that cellobiose detection may occur prior to internalization. However, only a few cases of nontransporting (putative) receptors have been reported in filamentous fungi. In addition, a novel cellodextrin transporter-like protein, Clp1, also forms a critical component in the regulation of cellulase production (36). However, Clp1 did not import cellodextrins, and actually suppressed the transcription of cellulases and other cellodextrin transporters in response to cellobiose or cellulose (36). Therefore, despite the knowledge of nontransporting receptors in filamentous fungi being elusive, they appear to exist and can function either as inducers or repressors of the lignocellulolytic machinery.

NONTRANSPORTING RECEPTORS SENSING NITROGEN (AMINO ACIDS, AMMONIUM, OTHER SOURCES)

Nitrogen is essential for the synthesis of many cellular components (amino acids, nucleotides, …). Therefore, cells developed a whole series of systems to sense and take up these nutrients. Apart from plasma membrane localized systems, discussed in this review, also the target of rapamycin complex 1 (TORC1) is a major growth regulator in response to nitrogen quality and

quantity. This is described in detail in a review on target of rapamycin (TOR) signaling (183).

Amino Acid Sensing by Ssy1 in *S. cerevisiae*

In *S. cerevisiae*, amino acids are detected at the plasma membrane by a multiprotein complex composed of Ssy1, Ptr3, and Ssy5 (SPS) (37) (Fig. 2). This results in induced expression of amino acid transporter genes as well as other nitrogen-metabolizing enzymes (38). Ssy1 is a unique member of the amino acid permease (AAP) family and is the receptor of the SPS (Ssy1-Ptr3-Ssy5) complex (39). Although Ssy1 does not transport amino acids, it does undergo a conformational change upon binding of its ligand (40). Direct binding of the amino acid to Ssy1 was proven by the identification of hyper- and hyporesponsive mutants (41, 42). The extremely long Ssy1 N-terminal tail is highly conserved in other yeast species, and it is required for its function as a sensor-signal transducer (43).

Ptr3, the least understood component of the complex, is induced by Yck1/2-dependent phosphorylation (44). Recent results indicate a key role for Ptr3 as an adaptor protein, linking the amino-acid-induced conformational change of Ssy1 to Yck1/2-Ssy5 access (45). Ptr3 multimerization, a well-established concept for G-protein-coupled receptors (GPCRs) as well as receptor tyrosine kinases (46, 47), occurs via the WD40-like domains, and is essential for downstream activation.

The chymotrypsin-like protease Ssy5 is synthesized as two domains, a catalytic domain and an inhibitory prodomain. Autocatalytic cleavage occurs during synthesis; however, the parts remain associated forming an inactive complex (48). In the presence of amino acids, the prodomain is released, leading to activation. The release of Ssy5 requires phosphorylation by type I casein kinases Yck1/2 followed by ubiquitination by SCFGrr1 (49, 50). Ubiquitination leads to proteasomal degradation of the prodomain, yet the direct involvement of the 26S proteasome has been questioned (50). The Ssy5 prodomain contains a phosphodegron, which may represent the site of Yck1/2 recognition (51). This sequence was also found in Mth1 and Std1, effector components of the Snf3/Rgt2 glucose-sensing pathway that also requires Yck1/2 and SCFGrr1 (52).

The amino-acid-induced receptor-activated proteolysis (RAP) of Ssy5 results in endoproteolytic removal of the N-terminal tail of the transcription factors Stp1/2, which are synthesized as latent cytoplasmic precursors (53). The processed forms of Stp1/2 are translocated into the nucleus where they bind to, and induce the expression of, their target genes. Although homologous, Stp1/2 are functionally divergent, possessing a different N-terminal tail, and also showing differential degradation and distinct cellular localization, providing a mechanistic explanation for Stp2 being the primary activator (54, 55).

Several control levels to maintain the derepressed state of signaling in the absence of inducing amino acids exist. First, Ssy5 activity is controlled by its Rts1/PP2A-dependent dephosphorylation, which antagonizes the Yck1/2-dependent phosphorylation of the prodomain, and thereby mutes Ssy5 activity in the absence of amino acids (44, 45, 56). Second, the Asi1-3 inner nuclear membrane proteins restrict entrance of full-length Stp1/2 in the nucleus because of its N-terminal region (57, 58). A third level of regulation is the targeting of unprocessed Stp1 for degradation, thereby diminishing its nuclear abundance (59). An additional level of Stp1 regulation occurs by TOR; inactivation of TOR by rapamycin results in the degradation of Stp1 in the nucleus, which is dependent on the phosphatase 2A (PP2A)-like phosphatase, Sit4 (60). All these control systems show that the cells have invested a lot in order to have activation of the pathway only in conditions where this is relevant. More information on TOR signaling is presented in reference 183.

Amino Acid Sensing in *C. albicans* and *C. neoformans*

The transcriptional regulation of AAP genes by the presence of their substrates in *C. albicans* occurs in a manner similar to that of *S. cerevisiae* (61). The *Candida* genome encodes homologs of all characterized components of the SPS complex (62). *Ca*Csy1, the *Sc*Ssy1 homolog, similarly acts as the primary receptor (Fig. 2). Despite the high degree of homology, the N terminus of both proteins is very divergent, potentially contributing to their differences in specificity (61). *C. albicans* cells lacking *Ca*Csy1 show altered morphology and hyphal formation, indicating a role in amino acid (serum)-induced hyphal morphogenesis.

Similar to *S. cerevisiae*, the sensor complex induces proteolytic processing of two latent transcription factors, *Ca*Stp1/2, upon amino acid detection (63). Both proteins are activated upon amino acid addition, to differentially activate two modes of nitrogen acquisition: *Ca*Stp2 activates genes required for amino acid uptake, while *Ca*Stp1 induces genes involved in extracellular protein degradation and peptide uptake. In addition, the levels of *Ca*Stp1 itself are also amino acid regulated, indicating that *Candida* cells preferentially use amino acids (63). Recent results indicate that the control of *STP1* expression is mediated by the two GATA

transcription factors, *Ca*Gln3 and *Ca*Gat1 (64). With the use of the insect host, *Drosophila*, it was shown that a functional SPS sensor and proper Stp1 gene control are required for host protein catabolism and utilization, while being essential for full virulence. *Ca*Stp2, on the other hand, is dispensable for virulence (65).

Recently, eight global affinity amino acid permeases and two methionine permease homologs have been identified in *C. neoformans* (66). From this low number of permeases, it is apparent that this pathogen is more dependent on amino acid biosynthesis than on uptake. Transcriptional profiling revealed that these genes are subjected to regulatory mechanisms known to respond to the nutritional status in other fungi, such as nitrogen and carbon catabolite repression and SPS sensing as well as general amino acid control. Inhibitors of these pathways caused *in vitro* growth arrest. Despite the presence of *ScSSY1* and *ScSTP2* homologs, an SPS sensing pathway as in *S. cerevisiae* or *C. albicans* has never been described in *C. neoformans*.

To date, nontransporting amino acid receptors have not yet been described in filamentous fungi, while nontransporting ammonium sensors have not been described in any fungal species.

TRANSCEPTORS (TRANSPORTING RECEPTORS) SENSING CARBON SOURCES

Transceptor-Mediated Carbon Sensing in *S. cerevisiae* and Other Yeasts

In *S. cerevisiae*, none of the hexose carriers has been directly implicated in activating/repressing downstream signaling pathways. Glucose repression appears to be independent of plasma membrane transceptors (67). This is different from the situation in mammalian cells where transporters such as GLUT1, GLUT2, and GLUT4 have been shown to activate downstream signaling pathways (68–71).

Transceptor-Mediated Carbon Sensing in *C. albicans* and *C. neoformans*

The presence of carbon-sensing transceptors in *C. albicans* or *C. neoformans* has not been clearly demonstrated. In *C. albicans*, Hgt4 and Hgt12 have been reported to be transceptors that regulate glucose sensing and uptake (Fig. 1). Whether Hgt4 has glucose uptake activity remains untested, but Hgt12 seems to have glucose transport activity, potentially indicating that this may be a *bona fide* transceptor (21, 72, 73). Hgt12 is specifically expressed during macrophage infection, probably as a response to the absence of glucose in the phagolysosome (72). In *C. neoformans*, Hxs1 has high sequence identity with *Sc*Snf3/*Sc*Rgt2, yet it still shows glucose uptake activity (24). Mutagenesis showed that Hxs1 is required for efficient glucose uptake and fungal growth under low-glucose conditions. Hxs1 is not required for regulating the expression of hexose transporter candidates, and heterologous expression of *HXS1* does not complement the *S. cerevisiae snf3*Δ *rgt2*Δ phenotype. Therefore, whether Hxs1 also functions as a glucose sensor remains to be defined. Importantly, however, Hxs1 is required for fungal virulence in a murine infection model of cryptococcosis. Whether Hxs2 could function as a glucose sensor remains unclear. Interestingly, Hxs1 and Hxs2 do not have the long C-terminal tail that has been shown to be essential for sensory function of their orthologs in *S. cerevisiae* and *C. albicans*. Similarly, in the methylotrophic yeast *H. polymorpha*, the hexose transporter homolog Gcr1 also lacks the typical C terminus, but its regulatory role, along with its involvement in hexose transport, has been demonstrated (74). Therefore, a long C-terminal tail may not be essential for glucose sensing in some fungal glucose transceptors.

Transceptor-Mediated Carbon Sensing in Filamentous Fungi

In filamentous fungi, transceptors are proposed to perform roles in the detection and uptake of polysaccharides, which in turn influences how these fungi interact with their environment. A dual role in signaling and in sugar transport has been shown for *N. crassa* Rco3, lacking the unique C terminus, although it is not fully clear whether this protein has an active transport capacity (14, 75) (Fig. 1). Similarly, Hxt4 from the hemibiotrophic maize pathogen *Colletotrichum graminicola*, which has very low similarity to the *Sc*Hxt, *Sc*Snf3, or *Sc*Rgt2 receptors in *S. cerevisiae*, was shown to be a sensor of nutritional status (76). Recently, Hxt1 from *Ustilago maydis*, a ubiquitous pest of corn, was characterized as a high-affinity hexose transporter that also functions as a receptor, which is required for full virulence (77). First, overexpression of a mutant (R164K), resulting in constitutive signaling in Snf3/Rgt2, leads to downregulation of hexose transport under glucose deprivation and complete loss of virulence. Second, heterologous expression of high-affinity transporters *C. graminicola HXT3* and *Arabidopsis thaliana STP1* only complement the growth defect of an *hxt1* strain and not its decreased virulence. How exactly the signal is transduced downstream is not yet elucidated. However, *U. maydis* possesses a homolog of the membrane-bound casein kinase I (Yck1/2), but no

homologs of all the other downstream components of the *Sc*Snf3/*Sc*Rgt2 pathway have been identified.

Transporters of cellodextrins, the breakdown product of cellulose, have been identified and shown to be fundamental to the regulation of cellulase production. Two cellodextrin transporters, Cdt1 and Cdt2, from *N. crassa*, were shown to contribute to cellulose sensing, and mutants lacking both transporters were unable to induce cellulases in response to cellulose (78). Insertion of these cellodextrin transporters into *S. cerevisiae* along with a *N. crassa* β-glucosidase enabled cellobiose fermentation (79, 80), demonstrating their transporter activity. However, the impairment of cellobiose transport, by introducing point mutations into CDT-1 or CDT-2, did not greatly impact cellulase induction in response to cellobiose, suggesting that the cellodextrin transporters act as transceptors with dual functions in the transport and the detection of cellobiose (78). In *T. reesei*, Stp1, an MFS sugar transporter capable of transporting cellobiose, was identified. However, its absence enhanced cellulase production, demonstrating how Stp1 repressed cellulase production (34). Hence, these studies have provided novel insights into the detection of cellulose by filamentous fungi and how cellobiose transceptors can regulate the lignocellulolytic enzyme production.

TRANSCEPTORS SENSING NITROGEN (AMINO ACIDS, AMMONIUM, OTHER SOURCES)

Transceptor-Mediated Amino Acid Sensing in *S. cerevisiae*

In *S. cerevisiae*, the uptake of amino acids involves several general as well as specific transporters, all belonging to the AAP family (81). One of the general permeases is Gap1, a proton symporter catalyzing transport of all L-amino acids including L-citrulline, 4-aminobutyric acid, some D-amino acids, and toxic analogs as well as polyamines (82, 83) (Fig. 2). The expression as well as the activity of Gap1 are tightly regulated by both the quality and quantity of the nitrogen sources available. Transcriptional regulation occurs via the nitrogen catabolite repression pathway, by the transcriptional activators, Gln3 and Gat1, and inhibitors, Dal80 and Deh1 (84, 85). Fine-tuning of the amount of plasma membrane-localized molecules according to nitrogen availability is regulated by the coordinated action of the Npr1 kinase, the α-arrestins Aly1/2, as well as the ubiquitin ligase adaptors Bul1/2 leading to ubiquitination of Gap1 by the ubiquitin-ligase Rsp5, followed by endocytosis and degradation in the vacuole (86–89). Some amino acid analogs, however, trigger oligo-ubiquitination without endocytosis, indicating that an additional modification is required to initiate the internalization process (90). An additional level of regulation at the plasma membrane is reversible inactivation, due to a conformation change, upon binding of amino acids (91).

Upon studying the amino-acid-induced activation of the PKA pathway it was found that the well-known transporter Gap1 has an additional role as an amino acid receptor (92). The involvement of metabolism and intracellular sensing has been excluded in order to prove the direct receptor function. High L-citrulline concentrations do not trigger signaling in a *gap1*Δ strain, despite its transport by other carriers. The involvement of metabolism was excluded because nonmetabolizable D-amino acids trigger the same activation as their metabolizable L-counterparts. Moreover, deletion of the first enzyme required for L-citrulline catabolism does not abolish L-citrulline-induced activation. Direct binding of the agonists as well as identification of two residues, Ser^{388} and Val^{389}, exposed with their site chain in the binding site, was obtained by the substituted cysteine accessibility method (SCAM) analysis of the eight transmembrane domain (93). An additional strong argument that transport through the transceptor is not required to trigger signaling was the identification of nontransported signaling agonists. A recent study showed that, for both signaling and endocytosis, different substrates elicit specific conformational changes upon movement through the passageway, indicating that all these events (transport, signaling, or endocytosis) can occur independently of one another (90). Despite indications for the involvement of certain proteins in the downstream signaling, such as the PKA complex, the Sch9 protein kinase, and the *S. cerevisiae* PDK1 orthologs (Pkh1-3), the exact mechanism by which the Gap1 transceptor relays the signal downstream remains unclear (94, 95). For example, a basal level of cAMP is required, but there is no transient increase in cAMP upon amino acid activation (96).

Transceptor-Mediated Amino Acid Sensing in *C. albicans* and *C. neoformans*

C. albicans expresses six orthologs of the *S. cerevisiae GAP1* (97). Complementation experiments in the *Scgap1*Δ strain showed that *Ca*Gap1, 2, and 6 can also function as transceptors because they are able to rapidly activate the PKA pathway upon addition of their substrates to nitrogen-starved cells (97) (Fig. 2). Although the different *Ca*Gap permeases share high

sequence similarity, substrate specificity differs substantially. It remains to be determined whether these proteins also function as transceptors in *C. albicans*.

In *C. neoformans*, there are at least seven *Sc*Gap1 homologs in the H99 genome database. However, the function of this gene group has not been analyzed in detail. It remains unknown whether there is any transceptor-mediated amino acid sensing in *C. neoformans*.

Transceptor-Mediated Amino Acid Sensing in Filamentous Fungi

So far, no studies have shown the presence of transceptor-mediated amino acid sensing in filamentous fungi.

Transceptor-Mediated Ammonium Sensing in Yeasts

Ammonium is an important nitrogen source acquired from the breakdown of nitrogenous compounds or its specific uptake from the environment. A conserved family of transporters mediates ammonium import, and some additionally act as transceptors (98–104). When levels of easily metabolizable nitrogen sources are low, or when cells are grown on a poor nitrogen source, fungi experience nitrogen limitation, resulting in reduced growth. During this nitrogen limitation, the ammonium transceptors regulate mating in *C. neoformans* and filamentous growth in *S. cerevisiae*, *Schyzosaccharomyces pombe*, *C. albicans*, and *U. maydis* (98–104). The *S. cerevisiae* genome encodes three ammonium transporters (Mep1-3); however, only Mep2 is a transceptor, acting as a regulator of pseudohyphal growth and PKA pathway activation when ammonium is reintroduced to nitrogen-starved cells (98, 100, 105). Expression of the *MEP* genes is regulated by nitrogen catabolite repression, while the Mep2 protein is controlled by the TORC1-regulated Npr1 kinase (106, 107). Mep2 phosphorylation is not required for plasma membrane targeting and stability, but exerts a positive control on protein activity by initiating a conformational change in the cytoplasmic C-terminal domain of the protein (107, 108).

Ammonium transporters are homotrimers with each monomer forming a right-handed helical bundle that surrounds the pore through which ammonium translocation takes place. Substrate binding occurs at a discriminating extracellular site and two overlapping phenylalanine residues block the pore opening (108–112). Unlike bacterial homologs, the pores of Mep2 from both *S. cerevisiae* and *C. albicans* are closed on the cytoplasmic side of the protein (108). Molecular dynamics simulations support the deprotonation of the ammonium ion in bacterial transporters and the movement of ammonia gas and protons separately through the protein (113–116). A similar mechanism of transport may occur in the fungal transceptors because they contain two histidine residues (His_1-His_2) in the pore that may act as a proton relay system (108, 116). Nonsensing fungal ammonium transporters contain a glutamate and a histidine residue (Glu_1-His_2) at the equivalent positions, although the significance of this difference with regard to sensing has not been established (117). Sensing is dependent on ammonium import through the transceptor, and mutations have been identified that uncouple transport and signaling and that distinguish between PKA activation in starved cells and pseudohyphal induction (105, 118). The Mep2 His_2 residue is particularly interesting because it is dispensable for transport, but essential for signaling, and is involved in blocking the cytoplasmic side of the pore (108, 118). The potential importance of the His_2 residue in signaling is supported by mutation of an adjacent residue that generates a hyperactive sensor (107).

Transceptor ammonium sensing does not reflect changes in internal nitrogen metabolism, because the nonmetabolizable ammonium analog methylamine induces the rapid activation of the PKA pathway, and signaling mutants that import ammonium exist (105, 117). These findings suggest that it is the action of the protein as a transporter that is important for the sensing event. In one model the conformational changes that occur during the transport process could be channeled to a signaling partner. In support of this model, conformational changes occur in Mep2 in response to phosphorylation, and the closed nature of the pore suggests additional conformational changes might occur during ammonium transport (107, 108). Furthermore, a physical interaction has been identified between the Ump2 receptor and the signaling protein Rho1 in *U. maydis* (119). In *S. cerevisiae* and *C. albicans* genetic interactions between Mep2 and the PKA signaling pathway have been identified (100, 101, 118). It has also been proposed that signaling and nonsignaling transporters may influence internal pH differently if one mediates electrogenic rather than electroneutral transport (117). In this model, sensing would be dependent on a downstream pH sensor.

Transceptor-Mediated Ammonium Sensing in Filamentous Fungi

The number of ammonium transceptors that have been studied suggests that this mode of signaling may be widespread in fungi. To support this, homologs from diverse fungi restore signaling in a *S. cerevisiae* strain

lacking the Mep2 transceptor, including transporters from the filamentous rice pathogen *Fusarium fujikuroi* (MepA and MepC) and the ectomycorrhizal fungus *Hebeloma cylindrosporum* (Amt1 and Amt2) (120, 121). Although these proteins are able to restore pseudohyphal growth in *S. cerevisiae*, the processes they regulate within their native organism remain unclear. In the broad-range plant pathogen *Colletotrichum gloeosporioides*, transceptor-mediated ammonium sensing has been shown to be important for invasive growth and virulence (122).

G-PROTEIN-COUPLED RECEPTORS

GPCRs communicate changes in the extracellular environment to intracellular G proteins, which initiate signaling events that coordinate the appropriate response. GPCR-ligand binding causes the dissociation of the heterotrimeric G proteins into the Gα-subunit and a Gβγ-dimer, which activate downstream pathways (123). G-protein functions are transient and regulated by repressors of G-protein signaling (RGS), which promote G-protein reassociation and RGS ubiquitination/degradation feedback mechanisms. Despite the general GPCR-signaling mechanism being highly conserved in eukaryotes, significant differences exist between yeasts and filamentous fungi. Five main categories of classical GPCRs exist, including pheromone, carbon, nitrogen, cAMP, and microbial opsin receptors. Additional nonclassical GPCRs include the orthologs of Pth11 (*Magnoporthe oryzae*), mPR (humans), a hormone receptor (rat), and *At*Rgs1 (*A. thaliana*). However, relatively few GPCR-G-protein interactions in fungi are characterized, and even fewer pathway-activating ligands have been identified.

GPCR-Mediated Carbon Sensing in *S. cerevisiae*

The addition of various carbon sources, such as glucose, sucrose, mannose, or fructose, to glucose-deprived cells triggers rapid activation of the cAMP-PKA pathway (124–126). PKA becomes activated because of an increase in cAMP, as a result of adenylate cyclase activation via a dual-glucose-sensing system, extracellular detection through a GPCR, as well as intracellular sensing dependent on sugar uptake and phosphorylation (127) (Fig. 1). The activation of PKA results in the phosphorylation of a wide spectrum of target proteins associated with rapid cell growth (128).

Extracellular sugar sensing is mediated via the GPCR, Gpr1, the Gα-protein Gpa2 (129), and its RGS protein, Rgs2 (130). Detailed analysis of the *Sc*Gpr1 receptor, using cysteine-scanning mutagenesis and SCAM, identified specific amino acids in transmembrane domain 6 that bind glucose and sucrose as ligands, thereby mediating the activation of the PKA pathway (125).

In addition to a role in activation of the PKA pathway, Gpr1 is required for pseudohyphal filamentous growth and also accounts for the glucose sensitivity of the mating pathway (131). The Sks1 kinase was recently identified as a novel component linking glucose sensing by Gpr1 and induction of genes involved in pseudohyphal differentiation (132). Gpa2 is an atypical Gα-protein, in that it is not part of a heterotrimeric complex; however, some proteins have been identified as candidate Gβγ-subunits: the Kelch repeat proteins, Krh1/Gpb2 and Krh2/Gpb1 (133, 134), as well as Asc1, a protein binding to Gpa2 in a guanine nucleotide-dependent manner (135). The Krh proteins, activated by Gpa2, downregulate PKA by increasing the cAMP dependency (136). In this way, they are part of an adenylate cyclase bypass, allowing the direct activation of PKA by activated Gpa2. In addition, the Krh proteins provide a negative feedback loop by phosphorylating the PKA regulatory subunit, Bcy1, and hence promoting its association with the catalytic subunits (137, 138). Recently, Gpr1 was also shown to be required for the induction of the mating pathway (139).

GPCR-Mediated Carbon Sensing in *C. albicans* and *C. neoformans*

In *C. albicans*, the situation regarding the number of GPCRs is similar to that of *S. cerevisiae*. There are two mating-factor GPCRs and one ortholog of the *ScGPR1*, *Ca*Gpr1 (140–142). Deletion of *CaGPR1* results in a strong morphogenetic defect on hyphae-inducing media, less damage in a reconstituted tissue infection model but only a slight effect on virulence in a systemic infection model system (141) (Fig. 1). Contrary to the situation in *S. cerevisiae*, *Ca*Gpr1 is not required for the glucose-induced cAMP increase. Although the exact ligand still remains to be identified, it is possible that *Ca*Gpr1 is a glucose sensor that does not activate adenylate cyclase. Another possibility is that methionine is the ligand, because it was shown that *Ca*Gpr1 is internalized in the presence of methionine (resembling the ligand-induced internalization known in other receptors) and there is no methionine-induced morphogenesis in the *Cagpr1* mutant (141, 143). This latter phenotype is only observed in the presence of low physiological concentrations of glucose, however, so it cannot be excluded that *Ca*Gpr1 is a low-glucose sensor.

In *C. neoformans*, glucose is a preferred carbon source and a known signal to activate the homologous

Gpa1-cAMP-PKA signal transduction pathway, which controls the production of major virulence factors (capsule and melanin) and is essential for fungal virulence (144). But it remains unknown how glucose activates Gpa1 and what is the GPCR for glucose sensing in *C. neoformans*. Multiple GPCR candidates have been identified based on the basic seven-transmembrane domain structure, but none of them share extensive sequence identity with *Sc*Gpr1, except maybe Gpr4 (145). Both Gpr4 and *Sc*Gpr1 have a large third cytoplasmic loop and a long C-terminal tail. Despite the structural similarity, Gpr4 is not important for glucose sensing, but, as mentioned below, is classified as a methionine receptor. Gpr5 also contains a large third intracellular loop and shares high sequence identity with Gpr4, and Gpr5 was found to play a role in regulating fungal cell size ("Titan" cell formation) during infection, likely by sensing certain unidentified host signals in the lung (146). Phylogenetic analysis based on protein sequences shows that Gpr4 and Gpr5 are more closely related to GprH in *A. nidulans* and cAR1 in *Dictyostelium discoideum* than to Gpr1 in *S. cerevisiae* (145). This is also consistent with the data indicating that Gpr4 and Gpr5 are not glucose receptors. How glucose is sensed remains to be understood. It is possible that there is functional redundancy among GPCRs in glucose sensing that complicates the identification of glucose receptors.

GPCR-Mediated Carbon Sensing in Filamentous Fungi

In contrast to yeasts, filamentous fungi can utilize a diverse array of nutrient sources including hexose, pentose, and complex saccharides. This nutritional plasticity may therefore be reflected in the presence of additional Gα-proteins and the dramatic expansion of putative GPCRs (123).

In *N. crassa*, the putative carbon sensor, Gpr4, interacts with the G protein, Gna1, influencing cAMP production and asexual development in a carbon source-dependent manner (147) (Fig. 1). The absence of Gpr4 resulted in reduced growth on glycerol, mannitol, and arabinose, a phenotype that was partially recovered via the addition of exogenous cAMP. In *A. nidulans*, a putative carbon receptor, GprD, influences hyphal growth, germination, and primary metabolism (148). Interestingly, GprD and the pheromone receptor, GprB, possess opposing functions in the regulation of sexual development (149). Similarly, the *A. nidulans* cAMP receptor-like GPCR, GprH, was induced during carbon starvation and was shown to be involved in glucose and amino acid sensing, while influencing primary metabolism via the cAMP-PKA pathway and acting as a repressor of sexual development (150). In *Aspergillus fumigatus*, two putative carbon receptors, GprC and GprD, were fundamental to germination, hyphal extension, and branching (151). Furthermore, the absence of GprC or GprD increased sensitivity toward environmental stress caused by reactive oxygen intermediates and displayed attenuated virulence in a murine infection model. Transcriptome analyses indicated that both GprC and GprD promoted primary metabolism, while inhibiting secondary metabolism (151). A wider evaluation of the function of all 15 classical GPCRs in *Aspergillus flavus* revealed multiple GPCRs potentially involved in carbon sensing, as the individual absence of GprA, GprC, GprJ, GprK, or GprR impaired growth on several carbon sources (152). Therefore, in contrast to yeasts, filamentous fungi appear to possess multiple GPCRs involved in sensing a broad range of carbon sources, reflecting the complexity of the environments that they inhabit, their metabolic plasticity, and their capacity to utilize a diverse array of carbon sources.

GPCR-Mediated Amino Acid Sensing by GPCRs in *C. albicans* and *C. neoformans*

Apart from the above-mentioned amino acid receptors, some GPCRs have also been reported to sense amino acids in fungi. In *C. albicans*, Gpr1, the homolog of the glucose sensor *Sc*Gpr1 has been suggested as a possible receptor for methionine. Methionine is important for the yeast-to-hyphal transition on solid agar medium; the *gpr1*Δ/*gpr1*Δ mutant blocks fungal filamentation under such inducible conditions. Gpr1 senses methionine to activate the cAMP-PKA pathway, which in turn controls the filamentation in the presence of glucose as a carbon source (141, 143). It is not yet excluded, however, that *Ca*Gpr1 may actually sense low (or physiological) glucose levels.

In *C. neoformans*, the GPCR protein Gpr4 also plays a role in sensing amino acids to activate cAMP-PKA signaling (145) (Fig. 2). Mutagenesis demonstrated that Gpr4 is required for capsule production and fungal mating, two cellular functions controlled by the Gpa1-cAMP-PKA pathway. The direct interaction between Gpr4 and Gpa1 G protein has also been confirmed in a membrane-based yeast two-hybrid system. Methionine can trigger Gpr4 internalization and induces a transient cAMP production in a Gpr4-dependent manner. In addition, a low level of methionine can induce mating hyphal elongation in *C. neoformans*, which is blocked by the deletion of *GPR4*. These data indicate that Gpr4 may function as a receptor for amino acids, such as

methionine (145). However, the direct binding of amino acids to *Ca*Gpr1 or *Cn*Gpr4 has not been observed. Whether these GPCRs sense extracellular amino acid, intracellular amino acid, or both, also remains to be determined.

GPCR-Mediated Amino Acid Sensing in Filamentous Fungi

GPCRs in filamentous fungi have been reported to sense amino acids. The cAMP receptor-like GPCR in *A. nidulans*, GprH, shows distant homology to the cAMP receptors of *D. discoideum* and *A. thaliana*, as well as to *Gpr4* in *C. neoformans* (123). Nonetheless, GprH was revealed to be a putative receptor for both glucose and the amino acid tryptophan, forming part of the cAMP-PKA pathway and influencing primary metabolism, while repressing sexual development, during carbon starvation (150, 153). GprH also shows homology to the putative *N. crassa* carbon receptor, Gpr4. The absence of this receptor caused defects in perithecial development and ascospore discharge. However, the binding of this putative ligand was not defined (154). Therefore, the function of cAMP receptor-like GPCRs in regulating sexual development appears to be conserved in fungi, while receptors of this class may dually detect both sugars and amino acids. Additionally, in *A. flavus*, the absence of the putative carbon receptors, GprC and GprD, which showed homology to the *S. pombe* glucose GPCR Git3, inhibited growth on the amino acid, proline (152). Hence, filamentous fungal GPCRs of several classes may potentially influence various amino acid and carbon nutrient-sensing pathways.

GPCR-Mediated Ammonium Sensing in Filamentous Fungi

In contrast to yeasts, very little is known of ammonium sensing in filamentous fungi. The *A. flavus* genome encodes four receptors that show homology to the *S. pombe* Stm1 sensor, which regulates cell cycle progression in response to nitrogen starvation (155). The absence of one of these receptors, GprR, impaired growth on ammonium chloride and the amino acid, proline (152). This again suggests that in filamentous fungi a single GPCR may play a role in multiple nutrient-sensing pathways.

GPCR-Mediated Host Sensing in Filamentous Fungal Pathogens

In filamentous fungi, GPCR-regulated signaling pathways include the cAMP-dependent PKA and the mitogen-activated protein kinase (MAPK) cascades, which regulate growth, metabolism, mating, morphogenesis, cell division, cell-to-cell fusion, chemotaxis, toxicogenesis, and virulence (156). It is becoming increasingly apparent that GPCRs can bind multiple ligands and, in turn, possess additional functions. The vascular root phytopathogen, *Fusarium oxysporum*, demonstrates chemotaxis toward specific nutrients (glucose, glutamate, and aspartate, but not glutamine, methionine, ammonium, or galactose) and host tomato plant root exudates, in particular, a class II peroxidase that is associated with root hairs that represent a potential site for invasion (157). Chemotropic sensitivity to the α-pheromone is far greater than nutrients, suggesting that the two responses are mediated by distinct mechanisms. Accordingly, mutants in the Fmk1 filamentous growth pathway are impaired in their response to glutamate or glucose, but not the α-pheromone, while mutants in the Mpk1 cell wall integrity pathway are impaired in their chemotaxis toward the α-pheromone, but not glutamate or glucose. The absence of Ste2/Pre2 pheromone receptor in *F. oxysporum* abolishes chemotropism toward the α-pheromone and tomato root exudates, while having a minor impact on virulence, demonstrating how Ste2 GPCR-mediated signaling, which was previously thought to be exclusively involved in pheromone sensing, is also involved in detecting host cues and promoting virulence (157).

In the foliar rice pathogen, *M. oryzae*, the Gαβγ-subunits, and the nonclassical GPCR, Pth11, sense surface hydrophobicity and plant cutin monomers, regulating appressorium formation and virulence in a cAMP-dependent manner (158). Evaluation of the spatiotemporal dynamics of G-protein signaling during pathogenic development showed key components, including Pth11, MagA, Rgs1, and the adenylate cyclase, to be sequestered to the tubulovesicular network, where late endosomes controlled the geometry and activation/deactivation of the cAMP signal during pathogenesis (159). Subsequently, numerous Pth11-related receptors have been identified in *M. oryzae* and other filamentous fungi from the ascomycete subphyla, *Pezizomycotina*, including *A. nidulans*, *N. crassa*, and *Fusarium graminearum*, while none were found in other ascomycete subphyla, such as the yeasts or the basidiomycetes (123, 160). Hence, this nonclassical receptor class represents the most highly expanded receptor class in filamentous ascomycete fungi. In turn, a recent analysis of the classical and nonclassical GPCRs in *N. crassa* identified numerous Pth11 homologs and revealed their involvement in nutrient sensing. The absence of three Pth11 homologs in *N. crassa*, Gpr32, Gpr36, and Gpr39, enhanced growth on cellulose as

an alternative to sucrose (161). Therefore, in filamentous fungi Pth11-like receptors appear to participate in sensing plant-related compounds, applicable to a saprophytic and a pathogenic interaction with plants.

EXTRACELLULAR MUCIN RECEPTORS IN FUNGI

Extracellular Mucin Receptors in *S. cerevisiae*

Exposure of *S. cerevisiae* to nitrogen deprivation activates pseudohyphal and invasive growth, in addition to biofilm formation, via activating MAPK signaling to enable nutrient foraging and survival (162–165). Extracellular signaling mucins act as sensors of nutrient availability and physical contact, regulating the connection between cells or to a surface (Fig. 3). Fungal and human signaling mucins utilize a highly conserved induction mechanism that results in GTPase activation (166). Nutrient deprivation promotes the proteolytic cleavage of an extracellular inhibitory mucin domain, activating intracellular Msb2-mediated MAPK signaling (162, 167). The highly glycosylated extracellular mucin domain of Msb2 is cleaved off by the Yps1 yapsin protease, releasing the inhibitory domain into the surrounding environment to create mucous glycoprotein layers around cells, analogs of MUC1/2 mucins in humans (166, 167). Upon starvation, the Mig1 carbon catabolite repressor that inhibits alternative carbon usage is phosphorylated by Snf1, promoting its dissoci-

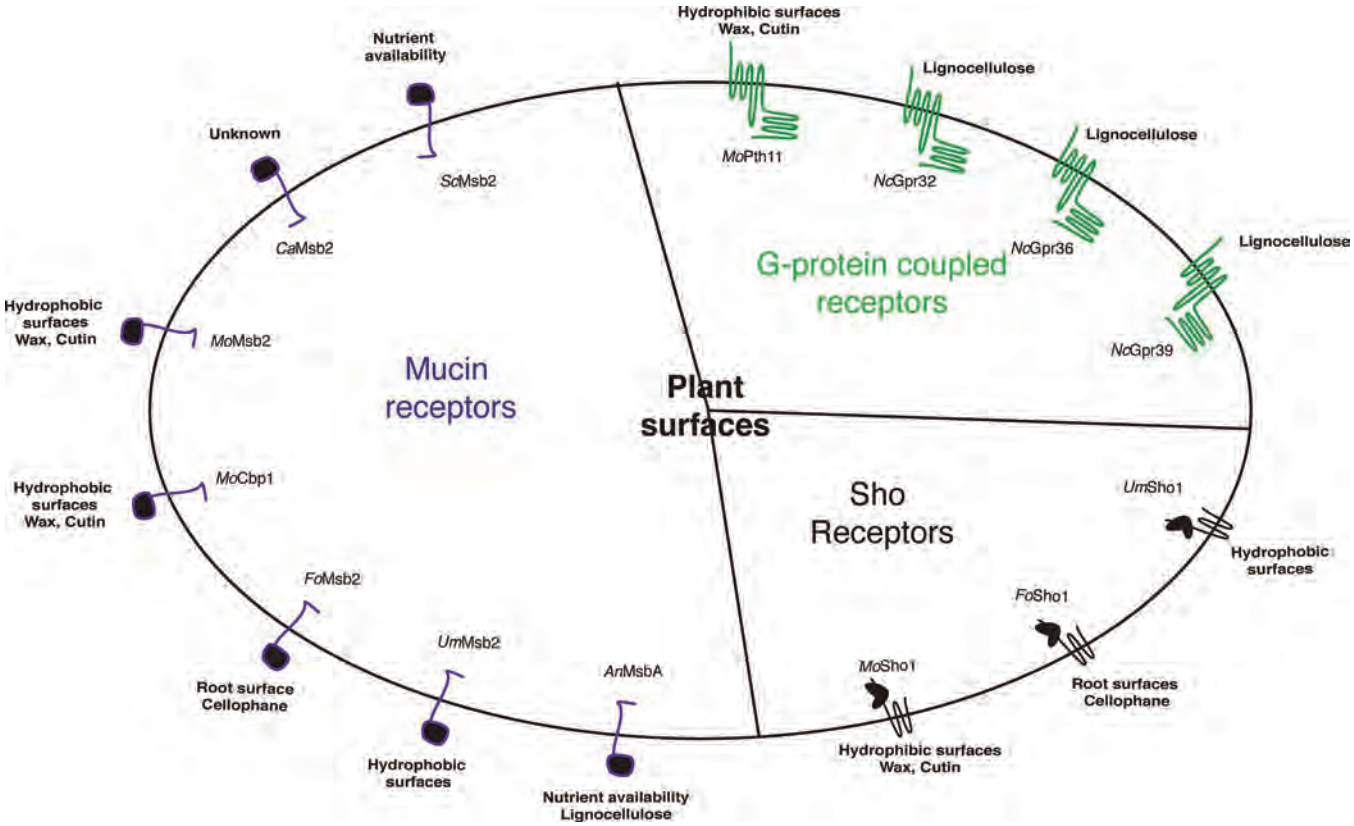


Figure 3 Surface-sensing proteins in the plasma membrane of fungal cells. Characterized surface-sensing proteins can be divided into G-protein-coupled receptors (GPCRs) and the interacting Sho receptors and the extracellular mucin receptors. The nonclassical, Pth11-type, GPCRs include the *Magnaporthe oryzae* (*Mo*) Pth11 receptor of hydrophobic surfaces and the *Neurospora crassa* (*Nc*) Gpr32, Gpr36, and Gpr39 putative lignocellulose receptors. The Sho receptors include the orthologous *Ustilago maydis* (*Um*), *Fusarium oxysporum* (*Fo*) and *Mo*, hydrophobic surface Sho1 receptors. The extracellular mucins include the orthologous *Aspergillus nidulans* (*An*), *Um*, *Fo*, *Mo*, Msb2 hydrophobic surface receptors, in addition to the Msb2 receptors of *Candida albicans* (*Ca*) and *Saccharomyces cerevisiae* (*Sc*) that have not been characterized as detecting surfaces, plus the additional Cbp1 mucin receptor of hydrophobic surfaces from *Mo*.

ation from the DNA, its migration from the nucleus, and its interaction with multiple members of the pseudohyphal MAPK cascade, including Msb2, which influences filamentation (164). The Snf1-Mig1 mechanism for preferential carbon usage is therefore interlinked to the pseudohyphal MAPK pathway, coordinating cellular metabolism and proliferation in response to nutrient availability (164). Cell adhesion is also controlled by the Ras, cAMP-PKA, and MAPK pathways in response to nutrient deprivation and other exogenous stresses (168), while adhesion impacts pseudohyphal growth and biofilm formation (169, 170). The Msb2-pseudohyphal MAPK pathway regulates the secretion of the Flo11 flocculin, which is essential for adhesion and pseudohyphal growth (169). Therefore, in response to nutrient availability, Msb2-MAPK signaling induces filamentous growth, cell adhesion, and biofilm formation to promote nutrient foraging and survival.

Extracellular Mucin Receptors in *C. albicans*

The regulation of MAPK cascades by Msb2, controlling filamentous growth, is conserved in *C. albicans*, where shedding of Msb2 and the induction of MAPK signaling promote filamentous growth and influence biofilm formation (171, 172) (Fig. 3). In *C. albicans*, Msb2, in cooperation with Sho1, controls the activation of the Cek1 MAPK under cell wall or osmotic stress. The absence of Msb2 had a stronger impact on invasive growth on certain solid media, such as mannitol or sucrose, and under hypoxia than the loss of Sho1 (171). Similar to *S. cerevisiae*, in *C. albicans* the Sap8 aspartic protease cleaves the extracellular Msb2 mucin domain, activating Cek1 signaling and biofilm formation (172). The absence of Msb2 or Sap8 resulted in the increased exposure of β-glucans and the loss of Msb2-attenuated virulence in a murine model of oral candidiasis. Additionally, Msb2 shedding also protects against antimicrobial peptides (173, 174). Therefore, in addition to regulating invasive growth and biofilm formation, the Msb2 pathway in *C. albicans* performs an essential function in reducing the exposure of pathogen-associated molecular patterns that trigger host defenses and in increasing the tolerance of exogenous stresses such as antimicrobial peptides by creating a mucous layer. However, the nutritional and/or environmental signals that promote Msb2 signaling remain to be determined in *C. albicans*.

Extracellular Mucin Receptors in Filamentous Fungi

Surface recognition is one of the most critical processes for both saprophytic and pathogenic filamentous fungi. In the heterogeneous semisolid environment, naturally occupied by fungi, the majority of nutrients are locked away as insoluble biomass. Hence, fungi have to actively search for and attach to a desirable source of nutrients. Subsequently, during this foraging period, starvation and contact sensing are hypothesized to participate in the localization and deconstruction of complex nutrient sources. In the lignocellulolytic fungus, *A. nidulans*, the extracellular mucin, MsbA, influences growth under nutrient-poor conditions including those on lignocellulose. Through the modulation of both the cell wall integrity and filamentous growth MAPK cascades, MsbA regulates adhesion to solid surfaces, biofilm formation, and lignocellulolytic enzyme secretion (175), in particular, the CbhA cellobiohydrolase required to release cellobiose from lignocellulose and the induction of the lignocellulolytic machinery. Therefore, starvation and/or surface sensing represent a new dimension to the already multifaceted regulation of nutrient sensing, CAZyme production, and lignocellulose deconstruction (Fig. 3).

In the case of filamentous phytopathogens, the recognition of host surfaces, and their subsequent penetration, is paramount for the establishment of infection. The filamentous growth MAPK pathway is central to the establishment of invasive growth and virulence (156). In the vascular root pathogen, *F. oxysporum*, Msb2 induces the phosphorylation of Fmk1, which promotes cellophane and tomato root penetration, contributing to fungal virulence (176). Similarly, the *F. oxysporum* Sho1 receptor was also required for Fmk1 phosphorylation, extracellular pectinolytic activity, cellophane penetration, plant tissue colonization, and virulence (177). The dimorphic pathogen of maize, *U. maydis*, switches from budding to hyphal growth and the development of appressoria in response to the detection of the hydrophobic, waxy, plant surfaces. In *U. maydis* the interacting Msb2 and Sho1 receptors function upstream of the Kpp2-Kpp6 MAPK cascade, and are key to the development of appressoria in response to hydrophobic surfaces, in addition to priming the fungus for biotrophic development (178, 179). These examples of the overlapping functionality of Msb2 and Sho1 suggest a genetic interaction between the two receptors, which was noted in yeasts, is conserved in filamentous fungi.

In the foliar rice pathogen, *M. oryzae*, multiple surface sensors detect rice leaf surfaces, appressorium formation, and host invasion, including the aforementioned nonclassical GPCR, Pth11, plus the Msb2, Sho1, and Cbp1 receptors (158, 180, 181). Similar to Pth11, the absence of Msb2 significantly reduced appressorium

formation and virulence, while the absence of Sho1 only resulted in a slight reduction in virulence. The dual absence of Msb2 Sho1 resulted in an inability to respond to cutin monomers and an inability to form appressoria on artificial hydrophobic surfaces. Appressorium formation on hydrophobic surfaces, leaf waxes, or primary alcohols was more severely inhibited in the dual absence of Msb2 and Sho1 than in the absence of just Msb2. This suggests that the two interacting receptors possess overlapping functions, yet Msb2 is critical for sensing surface hydrophobicity and cutin monomers, while Sho1 appears to be important in recognizing rice leaf waxes. Therefore, in addition to surface hydrophobicity and cutin monomers, primary alcohols in leaf epicuticular waxes are utilized as signals for fungal infection (180). *M. oryzae* possesses another putative mucin, CBP1 (named for chitin-binding protein 1), and the absence of Cbp1 also resulted in an inability to produce appressoria on hydrophobic surfaces, while retaining the ability to form appressoria on leaves (182), potentially because of the action of Msb2. In accordance, the absence of both the extracellular mucins, Msb2 and Cbp1, blocked Pmk1 MAPK phosphorylation, impeded appressorium formation, and abolished virulence, suggesting that the Msb2 and Cbp1 mucins have overlapping functions in the recognition of environmental cues required for the penetration of host tissues (181). Therefore, in filamentous fungi the extracellular mucin and Sho1 receptors appear to have evolved the ability to detect the hydrophobic, waxy, alcohol composition of plant surfaces and, in doing so, perform a fundamental role in plant pathogenesis.

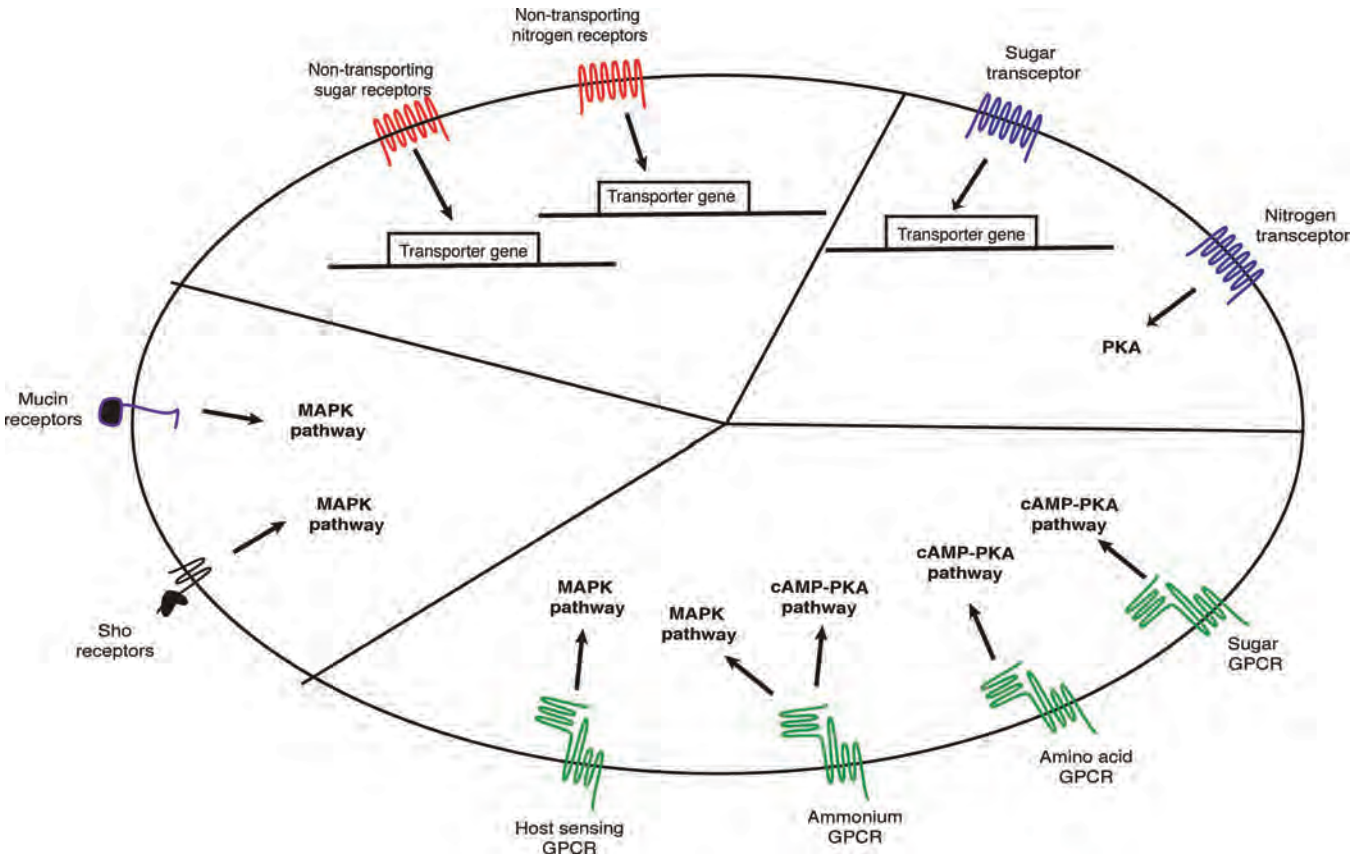


Figure 4 Nutrient- and surface-sensing proteins in the plasma membrane of fungal cells and the downstream signal transduction pathways they trigger. Examples for the different classes of nutrient (nontransporting receptors, transceptors, and GPCRs) and surface (mucin and Sho) sensing proteins are shown. Sensing of nutrients by nontransporting receptors and sugar transceptors results in the induction of nutrient transporter genes. Nitrogen transceptors (e.g., *Sc*Gap1) result in the activation of the PKA pathway upon sensing appropriate nitrogen sources. Depending on the type of GPCR, they can activate the cAMP-PKA pathway, the MAPK pathway, or both. The surface receptors activate the MAPK pathway.

MEMBRANE-LOCALIZED TRANSCEPTORS OR RECEPTORS LINK THE AVAILABILITY OF NUTRIENTS TO DOWNSTREAM SIGNAL TRANSDUCTION PATHWAYS

In this review, we described the nature of the receptors by which different nutrients are sensed by fungi. We distinguished between nontransporting sugar or nitrogen receptors, sugar or nitrogen transceptors, sugar, nitrogen, and host sensing GPCRs, as well as mucin receptors. In our review, we briefly mentioned the signal transduction pathways that are activated upon the sensing of nutrients by these receptors. Figure 4 gives an overview of what is currently known about the downstream signaling pathways, which we also briefly touched upon. The two most important downstream pathways are the MAPK and cAMP-PKA pathways, which are not only involved in nutrient sensing but are also involved in mating (183). Nontransporting receptors and some transceptors regulate the expression of nutrient transporters upon sensing that specific nutrient. A detailed description of these pathways is discussed elsewhere.

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The Complexity of Fungal Vision

20

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INTRODUCTION

Sunlight, harvested by photosynthetic organisms (plants, algae, and some bacteria) and used to synthesize energy-rich molecules (sugars) from carbon dioxide and water, provides the energy required to sustain life on Earth. In addition, sunlight properties such as intensity, duration, polarization, and spectral composition are used as sources of information (1). Indeed, all forms of life are continuously obtaining and decoding information from their environment. In fungi sunlight, ranging from ultraviolet (UV) to infrared wavelengths, regulates a diversity of biological processes including circadian rhythms, morphogenesis, tropism, and synthesis of pigments, among others (reviewed in reference 1). UV light can be harmful, since DNA modification products of photochemical reactions may be transmitted to the next generation as a mutation. Visible light appears not only to provide early warning of the presence of impending UV radiation and further damage, but also seems to contribute to the capacity of these organisms to deal with abiotic stress in general (2–5). Thus, the ability of most fungi to perceive and respond to light has very likely contributed to their survival and fitness.

Light responses in fungi, some of which can be extremely rapid (e.g., phototropism), were observed more than 150 years ago, and light-induced alterations in fungal morphology have been described for hundreds of fungi (6, 7). Responses may be triggered by very low light and require only nanoseconds to minutes of exposure with a rate of photons that could be only 10^{-10} mol of photons m^{-2} (8–10). Long-term effects of light rely mainly on genetic reprogramming of the fungal genome and usually involve major changes in gene expression patterns. A major question is how such coordinated activation and repression of hundreds of genes is achieved. Photoreceptors are proteins that contain light-sensing chromophores that modulate their biological activity after photoreception (10). The first step of light perception is a physical reaction, the absorption of photons by an organic molecule followed by conformational protein changes and transduction into biochemical reactions such as phosphorylation events.

Few physical and many genetic, molecular biology, and biochemical analyses have provided detailed information on the mechanisms of fungal vision. Although many open questions remain, it becomes clear that light sensing is a rather complex and multifaceted process with a strong impact on most aspects of fungal life. Hence, although most results rely on studies of a few model organisms, foremost *Neurospora crassa* but also *Aspergillus nidulans*, *Phycomyces blakesleeanus*, and *Trichoderma atroviride*, the strong impact of light in these organisms prompted studies of other fungi with pathogenic or symbiotic lifestyles or with biotechnological potential. All these studies continuously improve our knowledge of light regulation and help to unravel common principles but also species- or lifestyle-specific impacts of light. As a consequence of the growing number of publications combined with a long history of the field, it would be impossible to give a comprehensive overview and discuss all papers related to fungal vision. Instead we will concentrate on recent findings and refer to older publications when necessary. For more information the reader is referred to references 10 through 17.

FUNGAL VISION: THE PHENOMENON

Light represents only a small fraction of the electromagnetic radiation produced by the sun that organisms use for photosynthesis, photomorphogenesis, or orientation

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in the environment. Human vision, for instance, detects wavelengths between roughly 400 and 700 nm. However, shorter wavelengths such as UV and X rays (which are rare in nature) are also important, due to their damaging effects. Thus, the reaction of organisms to light can be divided into reactions to damaging irradiation and reactions to visible light (as defined by the human eye). Whereas the sensing and reaction to damaging radiation seems obligated for life on Earth, the reaction to visible light could be optional. However, one important function of light sensing may be the adaptation to harmful irradiation because visible light is a reliable indicator of the presence of UV radiation during the day.

Most fungi are able to sense and respond to light, with only a few exceptions that lack any indication of genes for sensing light (Fig. 1). Those organisms include the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* and some dermatophytic pathogenic fungi such as *Microsporum*. An exception appears to be the fission yeast *Schizosaccharomyces japonicus*, in which a blue-light effect and the presence of blue-light photosensors have been reported. However, it has to be considered that *S. japonicus* is a dimorphic fungus, which can switch between yeast and hyphal form (18). It has been speculated that light sensing may be of special interest for multicellular fungi that constantly need to adapt to changing environments (16). Most fungi respond in one or another way to light, and it is important to mention that only in some cases have the analyses of light responses been done using defined wavelengths (i.e., light-emitting diodes or monochromatic filters) and strict control of fluence rates (19–23).

Development of Basidiomycetes fruiting bodies depends on, among other factors, light or light-dark cycles (24). Scientists noted 60 years ago that under laboratory conditions the mushroom *Cyathus stercoreus* required light to initiate fruiting body development (25). Later it was shown that *Coprinus macrorhizus* primordia formation and stalk development, as well as the timing of meiosis, were all light dependent (26, 27). Some mushrooms not only produce meiotically derived spores in their caps, but are also able to generate different asexual spores, named oidia or chlamydospores. Oidia formation occurs in monokaryons and dikaryons of *Coprinus cinereus* and is blue-light dependent in dikaryons but not in monokaryons (28). In the basidiomycetous dimorphic yeast *Cryptococcus neoformans*, blue light controls growth, development, and virulence (29, 30).

In ascomycetes, light also has a strong impact in morphological and physiological processes, including the regulation of conidial germination, hyphal branching, sexual and asexual development, and secondary metabolism. Blue light induces sporulation in many ascomycetes, such as *T. atroviride*, *Aspergillus ornatus*, and *Penicillium isariiforme* (31). In contrast, *Alternaria solani* and *Botrytis cinerea* produce conidiophores under starvation conditions and after induction with UV light, which—if kept in the dark—produce conidia. Blue light inhibits the last step, conidia formation (31, 32). Furthermore, the inhibitory effect of blue light can be reversed by red-light illumination, which again can be reversed by far-red light (33). These complicated photoconidiation experiments suggested the presence of several photoreceptors in *B. cinerea*. In *A. nidulans*, conidiation is also induced by blue and red light, which together reach the level of white-light induction (34). Hence, light controls the balance between asexual and sexual development in this fungus (35). In *Trichoderma reesei* sexual development occurs in light (36), whereas in *N. crassa* fruiting bodies can be produced in the dark, although the number of protoperithecia is greatly increased upon exposure to blue light (37). Additionally, the position of the perithecium neck is also light dependent (38), with necks oriented to the light source but randomly oriented in the dark (39). Light is also required to obtain the maximum number of conidia in *N. crassa*, with a 4-fold increase in the number of conidia obtained in the light over the number obtained in the dark (40).

Germination, growth, and secondary metabolism are also affected by light. In *A. fumigatus* and *A. nidulans* light inhibits spore germination (41, 42), whereas it promotes hyphal branching in *N. crassa*, *Tuber borchii*, *T. atroviride*, and the plant pathogen *Colletotrichum trifolii*. Therefore, colonies look more compact in the presence of light (40, 43–45). In *N. crassa* and *Fusarium fujikuroi*, light activates the biosynthesis of carotenoids in vegetative mycelia, resulting in hyphae with a deep-orange color (46, 47). In *A. nidulans* sterigmatocystin (an aflatoxin precursor) and penicillin production are regulated by light (34, 48, 49). The study of the circadian clock in *N. crassa*, a light-regulated phenomenon, led to an enormous stimulation of the entire research field (15, 50–52). Conidiation in *N. crassa* is governed by a circadian clock that can be entrained by light (53), and it turned out that a main component of the clock system is the blue-light photoreceptor WC-1 (50, 54, 55).

Within zygomycetes, light regulates many aspects of the biology of *P. blakesleeanus*, *Mucor circinelloides*, and *Pilobolus crystallinus*, including growth and growth direction of fruiting bodies (phototropism), the development of sexual and asexual reproductive structures, and the biosynthesis of the beta-carotene pigment (56). Light effects have also been described in

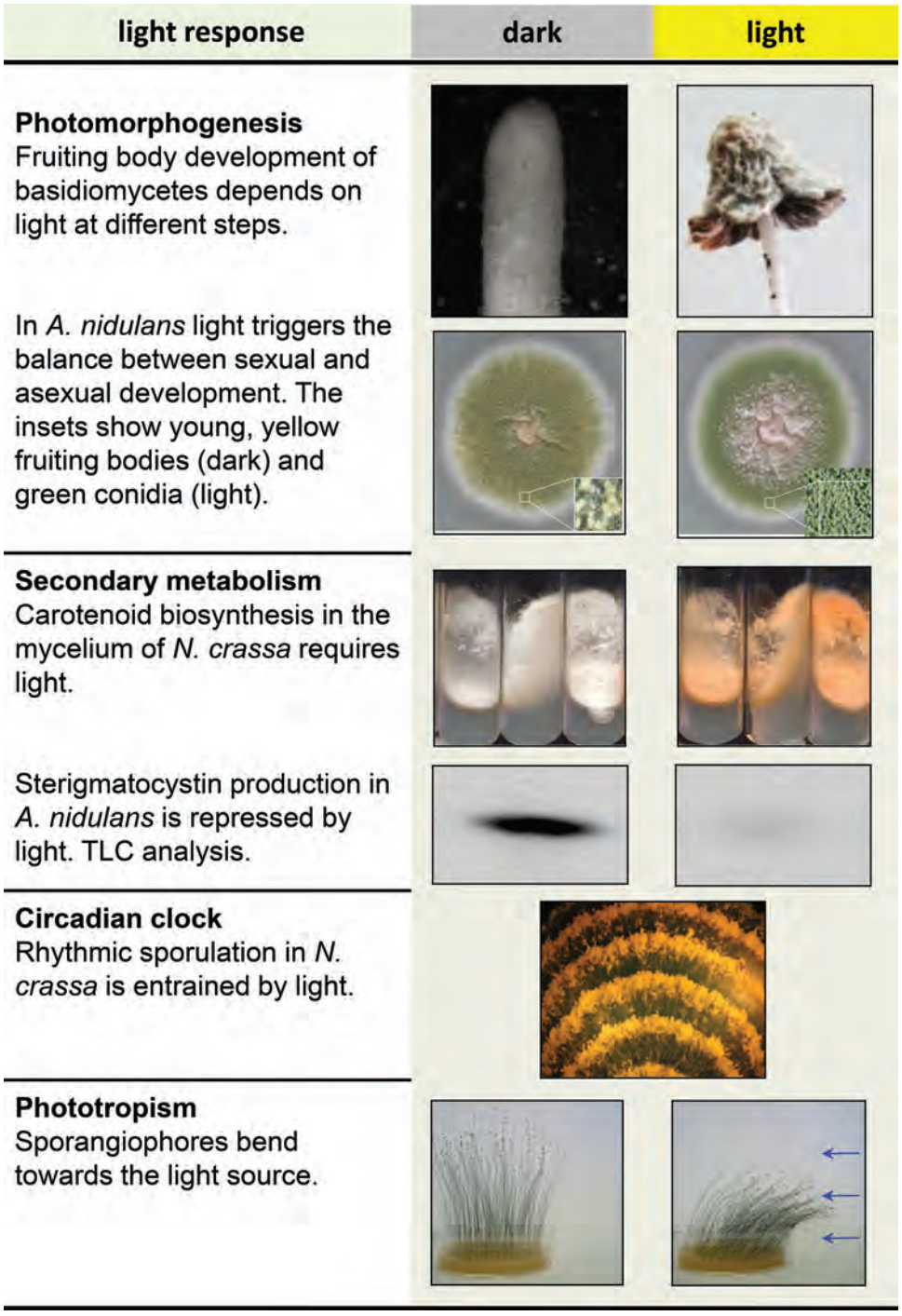


Figure 1 Phenomena of fungal responses to light. Light has a large impact on fungal morphology and physiology. The pictures of *Coprinus cinereus* were provided by Shanta Subba and Ursula Kües (University of Göttingen).

other basal fungi, including the regulation of zoospore mobility in the chytridiomycete fungus *Allomyces reticulatus* (57) and phototaxis in *Blastocladiella emersonii* (58). All these phenomena illustrate well the broad distribution of fungal vision and support the early evolutionary appearance of this feature.

The great variety of phenomena observed in response to light suggests that illumination causes a large reprogramming of the genetic machinery in fungal cells. Indeed, many genome-wide expression studies in various fungi show that hundreds of genes representing a significant fraction of the genome (5 to 10%) are differentially regulated by light (59–66). Whereas in animals one type of photoreceptor, rhodopsin, is used to sense blue, green, and red light, microbial eukaryotes and bacteria employ different classes of photoreceptors, each perceiving a narrow spectrum of light in the blue, green, or red part of the rainbow (Fig. 2). Here, we summarize the main properties of the classes of photoreceptors present in fungi.

PHOTOSENSORY PROTEINS AND THEIR MODES OF ACTION

The Flavin-Based White Collar Photoperception System

The search for components of the light perception machinery in fungi was first approached in *P. blakesleeanus*

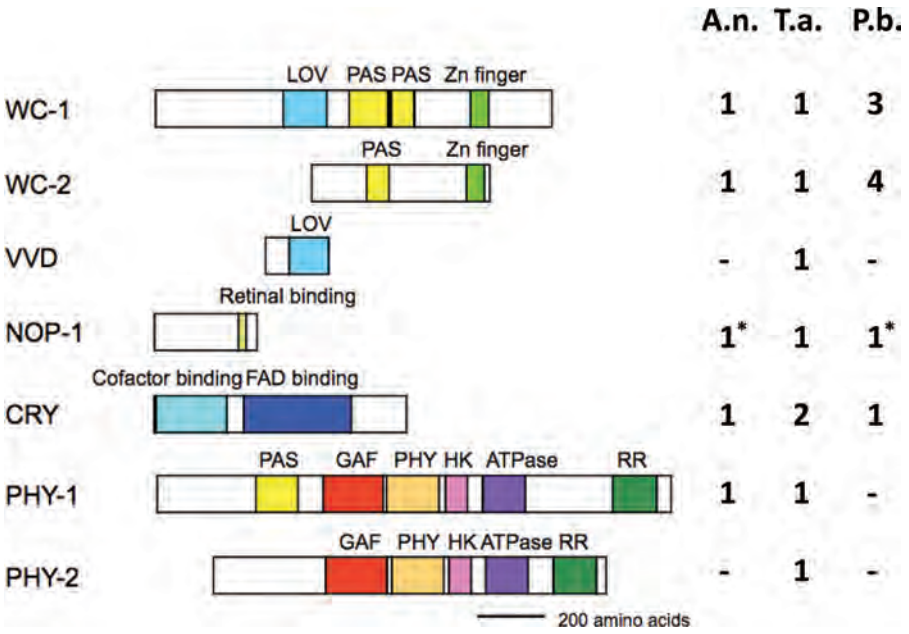


Figure 2 Schemes of the *Neurospora crassa* photoreceptor proteins and their presence in *Aspergillus nidulans*, *Trichoderma atroviride*, and *Phycomyces blakesleeanus*. The figure shows the set of *N. crassa* photoreceptors and a comparison of the presence of homologous genes in other model fungi, including the LOV-domain photoreceptors WC-1 and VIVID (VVD) together with WC-2, the protein that interacts with WC-1 to form the WC complex. Other photoreceptors identified in the *Neurospora* genome are a rhodopsin (NOP-1), a cryptochrome (CRY), and two phytochromes (PHY-1 and PHY-2). LOV-domain photoreceptors contain the flavin chromophore-binding domain (LOV) and may also contain the protein-interaction domains (PAS) and the Zn finger domain. Rhodopsins contain the retinal-binding domain. Cryptochromes contain the FAD chromophore-binding domain and the domain for binding the antenna cofactor. Phytochromes contain an amino-terminal sensory domain and a carboxy-terminal output domain. The sensory domain involved in binding the bilin chromophore is composed of three domains (PAS, GAF, and PHY). The output domain is composed of the histidine kinase domain (HK), the ATPase domain found in ATP binding proteins, and the response-regulator domain (RR) that is likely involved in relaying the light signal to other proteins. The number indicates the presence and number of photoreceptor protein encoding genes in the genomes of *A. nidulans* (A.n.), *T. atroviride* (T.a.), and *P. blakesleeanus* (P.b.). The * indicates that the protein is present but lacks the critical lysine residue required for retinal binding.

and *Trichoderma viride* (67–70). Max Delbrück and his research group described in detail the properties of *Phycomyces* responses to light and initiated the genetic study of the signal transduction pathways (68). Eventually, it was concluded that in both fungi the most likely receptor for blue-UV light was a flavoprotein (69–71). More than 30 years later the *T. atroviride* blue-light regulator gene (*blr1*) was cloned, corroborating the original conclusions reached based on action spectra (45). Blr1 belongs to a family of fungal blue-light receptors, whose founding member is the white collar-1 (WC-1) protein of *N. crassa* (72, 73). In *A. nidulans* the WC proteins are also conserved, although their role appears to be different because red light and phytochrome play dominant roles in light sensing (34, 74). Fungal photoreceptors belonging to the WC-1 family contain three PAS domains, the first of which is a LOV (light, oxygen, voltage) domain (55). LOV domains are specialized domains belonging to the PAS (Per Arnt Sim) superfamily and are present in proteins that sense UV-blue light in plants and fungi. They are about 110 to 120 amino acids in length and adopt a conserved α/β-fold that binds flavin dinucleotide (FAD) or flavin mononucleotide. Upon absorption of blue light, LOV domains undergo a photocycle, transiently forming a covalent flavin-thiol adduct with a conserved cysteine residue present in the LOV domain (75). The other two PAS domains are involved in protein-protein interactions (76). These photoreceptors also contain GATA zinc finger DNA binding domains, nuclear localization signals, and activation domains in several cases (10). However, there are exceptions to the presence of the activation domains, suggesting the involvement of additional proteins for the control of gene expression (1). The *N. crassa* WC-1 protein dimerizes with its partner, the WC-2 protein, which contains a PAS, a GATA zinc finger domain, and an activation domain to form the WC complex (WCC). Through Chip-Seq experiments, WCC has been found to directly control gene expression, functioning as a transcriptional factor (77). Only WC-2 is required for binding the WCC to DNA, whereas the zinc finger of WC-1 is needed for clock-related DNA regulation and DNA binding of the WCC. This suggests different mechanisms of gene regulation for light or clock-related processes (78). Mutants in either *wc-1* or *wc-2* are affected in all known blue-light responses. Similarly, mutants in the orthologs of these genes in other fungi are clearly affected in blue-light perception (1, 17).

As a product of genome duplication events, multiple WC genes are found in the *M. circinelloides* and *P. blakesleeanus* genomes (66). In *M. circinelloides*, three *N. crassa* WC-1 orthologous genes (*mcwc-1a*, *mcwc-1b*, and *mcwc-1c*) have been identified, all encoding LOV-domain proteins. *mcwc-1a* regulates phototropism, whereas *mcwc-1c* regulates photocarotenogenesis (79). The *mcwc-1b* gene product is also involved in carotenoid synthesis and is regulated by ubiquitylation by CrgA (80). In *P. blakesleeanus* three *wc-1* genes and four *wc-2* genes have been described (81, 82). The main key players in *P. blakesleeanus* photobiology are MadA and MadB, orthologs of WC-1 and WC-2, respectively, which form the main photoreceptor complex (81). Whole-genome transcriptome analysis carried out in *P. blakesleeanus* led to the proposal that the stage-specific transcriptional response to light relied on the expanded set of photoreceptors and other light-dependent transcriptional regulators that arose after genome duplication (66). In this regard, whole-genome duplication in vertebrates has resulted in specialization of genes for signal transduction that are part of their visual system (83). Thus, expansion of photoreceptors or downstream signal transduction genes has resulted in more elaborate photoperception systems in both vertebrates and fungi.

Some fungi contain secondary photoreceptors (Vivid and/or Envoy), consisting of a LOV domain and a few extra amino acids, and bind either FAD or flavin mononucleotide as a chromophore (84–86). These photoreceptors are important for photoadaptation under constant light and for responding to changes in light intensity. In *Neurospora*, vivid (VVD) fine-tunes light responses by interacting with the WCC through the WC-1 LOV domain by quenching these responses (87, 88).

Phytochrome: A Linear Tetrapyrrole-Containing Histidine Kinase

Phytochromes, first discovered in land plants, were later also found in algae, diatoms, bacteria, and fungi and are absent in animals (89–93). Phytochromes are composed of a multidomain protein with a linear tetrapyrrole covalently attached to the protein. The protein moiety consists of an N-terminal PGP or photosensory domain, which combines domains named PAS (found in Per, Arnt, and Sim), GAF (named for vertebrate cGMP-specific phosphodiesterases, cyanobacterial adenylate cyclases, and the transcription activator FhlA), and PHY (phytochrome-specific PAS-related). The chromophore attaches autocatalytically to a cysteine, located in most bacterial and fungal phytochromes in the PAS domain and in the GAF domain in the case of plants. The chromophore, a linear tetrapyrrole, absorbs red light and undergoes a conformational change,

which in turn changes its spectroscopic properties, and the absorption maximum is shifted toward far-red light. The conformational changes are reversible, and thus the chromophore interconverts from the red-light-absorbing Pr form to the far-red-light-absorbing Pfr form. The photosensory domain has been crystallized from bacterial phytochromes and recently from *Arabidopsis thaliana* (94–96).

The PGP domain is followed by a histidine-kinase domain, which consists of an ATP-binding and a substrate domain with a critical histidine residue. In plants this histidine residue is replaced by an arginine or glutamate, and thus the enzyme is not catalytically active. In contrast, in bacteria and fungi the histidine is conserved and phosphorylation activity has been demonstrated (91, 97, 98). In some bacteria and in fungi the histidine-kinase domain is followed by a response-regulator domain. Unlike plant phytochromes, *A. nidulans* FphA possess an N-terminal extension of 172 amino acids, which was shown to stabilize the Pfr form (97). The sequence similarities suggest a bacterial two-component signaling module as the origin of phytochrome and hence phosphorylation events as part of the signaling cascade (99, 100). In *A. nidulans*, *in vitro* experiments showed that the protein can become autophosphorylated at histidine number 770 and that it is capable of intermolecular phosphotransfer to the aspartate residue number 1181 of the response-regulator domain of a second phytochrome molecule (97). While autophosphorylation was stimulated by red light and shown to be dependent on the chromophore, transphosphorylation was independent of the chromophore and also found with apo FphA as the substrate. Autophosphorylation was stimulated with red light. In addition to the autophosphorylation activity of FphA, it was shown that aspartate 1181 serves as a substrate for YpdA, a histidine-containing phosphotransfer protein, *in vitro* (101). Hence, phosphorylation events are likely to play crucial roles in signal transduction in fungi and perhaps the interconnection with other pathways.

An open question concerns the nature and production of the chromophore. Although *in vivo* data are not yet available, it was shown that *A. nidulans* phytochrome incorporates biliverdin *in vitro* and, with less efficiency, phycyanobilin (74). Linear tetrapyrroles are normally generated by heme oxygenases. This is also how active phytochrome can be expressed in *Escherichia coli*, namely the coexpression of phytochrome along with a cyanobacterial heme oxygenase (74). However, heme oxygenase candidates were not found in the genome of *A. nidulans*.

Many fungi contain phytochromes, and sometimes even two or three genes can be found in their genomes (13, 102) (Fig. 2). However, functional studies remain to be conducted for most fungi. *N. crassa* contains two phytochrome genes, and it was only very recently that a role in the timing of sexual development could be assigned to one of them. Both phytochromes, though, affected the expression of a large number of genes (103). Thus, our knowledge of fungal phytochrome functioning is largely based on the results obtained in *A. nidulans*, and it is highly desirable that the light response of other fungi with pronounced red-light effects be studied at the molecular level. No phytochrome genes have been detected in the genomes of *P. blakesleeanus*, *M. circinelloides*, or other Mucoromycotina fungi, but a candidate phytochrome gene has been found in the chytrid *Spizellomyces punctatus* (Fig. 2) (17).

Cryptochrome and Photolyases

Cryptochromes are plant blue-light photoreceptors very similar to photolyases, enzymes required for blue-light-dependent DNA repair. They bind noncovalently the flavin chromophore FAD and other, probably secondary, chromophores (pterin or deazaflavin) and do not show photolyase activity (104, 105). Cryptochrome genes have been described in the genomes of several fungi, but their role in light sensing or DNA repair has been investigated only in some species (Fig. 2).

Neurospora CRY belongs to the cryptochrome-DASH subfamily, and the gene is induced by light in a WC-1-dependent manner and by the circadian clock (106). The *Neurospora* cryptochrome binds the blue light-absorbing chromophores FAD and MTHF (methenyltetrahydrofolate) when the gene is expressed in *E. coli* and shows DNA binding activity *in vitro* (106). A role has been proposed for CRY in the regulation of the activity of the WCC for the activation of some light-induced genes such as *con-10* (107). However, genome-wide activation of transcription by light is not significantly altered in the *Neurospora cry* mutant (106), suggesting a very limited role of CRY in the regulation of transcription by light. CRY also plays a marginal role in the regulation of the *Neurospora* circadian clock as a component of a secondary oscillator (108).

F. fujikuroi cryptochrome CryD is also a member of the CRY-DASH family and participates in the regulation of secondary metabolism and the development of conidia (109). In the ascomycete *Sclerotinia sclerotiorum*, deletion of the CRY-DASH gene results in minor developmental defects, suggesting that, as in *Fusarium*, this protein may have a sensory role in this fungus (110).

A. nidulans CPD photolyase CryA is phylogenetically related to cryptochromes and has dual roles as DNA repair enzyme and photoreceptor. CryA provided DNA repair activity in *E. coli*, and deletion of the *cryA* gene reduced the sensitivity in the near-UV/blue region of the spectrum for the inhibition of sexual development, supporting its role in this process (19). A similar dual role has been proposed for *T. reesei* Cry1, a member of the 6-4 photolyase family. Cry1 provides DNA repair activity in conidia of *T. reesei*, and its biochemical characterization shows that it can bind and repair DNA lesions *in vitro* or in *E. coli* cells expressing *cry1* (111). The changes in the pattern of light-dependent transcription in the *cry1* mutant suggested a sensory and regulatory role in the regulation of light-induced transcription (64).

A role in DNA repair has been proposed for a cryptochrome in *P. blakesleeanus*. This fungus shows a blue-light-dependent photoreactivation that is typical of photolyases despite the absence of a photolyase gene in the genome (112). Indeed, cryptochrome-DASH (CryA) provides DNA repair activity *in vivo* when expressed in *E. coli* and can bind and repair single-stranded and double-stranded DNA *in vitro*, suggesting that CryA acts as a photolyase for DNA repair in *P. blakesleeanus* (113). As such, CryA seems to represent an early step in the evolution of cryptochromes from DNA repair enzymes to sensory photoreceptors. It is tempting to speculate that proteins homologous to CryA in fungi related to *P. blakesleeanus* play similar roles as DNA repair enzymes despite being identified as cryptochromes by their amino acid sequences (113).

Opsins

Rhodopsins are membrane-embedded seven-transmembrane helix photoreceptors composed of a retinal chromophore bound to an opsin apoprotein (114–116). Despite their ubiquitous role as photoreceptors for animal vision, the opsin genes described in several fungi and their role in fungal photobiology are only starting to be appreciated.

Neurospora NOP-1, the first example of a fungal opsin, shows a slow photocycle and long-lived intermediates, consistent with a role as a sensory photoreceptor (117–121). However, the inactivation of *nop-1* did not result in a blind phenotype (120). *nop-1* mRNA accumulates during asexual or sexual development, with a large amount of the mRNA observed during late conidiation but not during early vegetative growth (120). A regulatory role for the NOP-1 protein has been suggested based on changes in mRNA accumulation of light and conidiation-regulated genes (122).

A rhodopsin gene has been isolated from *Leptosphaeria maculans*, the fungus responsible for blackleg disease of *Brassica* species. The *Leptosphaeria ops* gene seems to be constitutively expressed, unlike its *Neurospora* homolog (123), and is active as a light-driven proton pump when expressed in yeast membranes (124). The capacity of *Leptosphaeria* rhodopsin to act as a proton pump seems to require specific amino acids and the presence of hydrogen-bonded water molecules (119). However, the role of a light-dependent proton pump in the biology of *L. maculans* remains to be clarified.

Two opsin genes, *carO* and *opsA*, have been identified in the genome of *Fusarium* species. The gene *carO* was identified in a cluster of genes for carotene biosynthesis, suggesting a common regulation. Indeed, *carO* was induced after light exposure and was overexpressed in carotene-overproducing strains, like other neighboring *car* genes (125). Since the synthesis of the retinal chromophore requires a regular supply of carotenes, the coregulation of the opsin apoprotein gene with the genes involved in carotenoid biosynthesis may ensure that opsin apoproteins are not wasted in the absence of retinal molecules. CarO is a light-driven proton pump that is abundant in conidia and retards germination under light, suggesting a role for CarO in the regulation of fungal germination (126). The other opsin gene, *opsA*, is not linked to the *car* cluster and is subject to a different regulation: mRNA levels are moderately induced by light, and *opsA* is not overexpressed in carotene-overproducing strains (127).

In *A. nidulans* an opsin homolog can be found in the genome as well. However, the critical lysine residue for retinal binding is absent, and thus it is not yet clear if it is a chromoprotein and plays any role in light regulation. A similar opsin-like gene has been identified in the genome of *T. atroviride*, but its role remains to be identified. Opsin genes have not been detected in the genomes of *P. blakesleeanus* or *M. circinelloides* (Fig. 2) (17).

A detailed characterization of the role of an opsin as a fungal photoreceptor has been described for *B. emersonii*. The photoreceptor for phototaxis in this fungus is a fusion protein containing an opsin domain and a guanylyl cyclase catalytic domain, suggesting a role for the cGMP signaling pathway in vision as it has been described in vertebrates (58). It is tempting to speculate that this is an ancient mechanism of vision that was lost in most fungi and is still observed in vertebrates.

The absence of obvious phenotypes in most fungal strains lacking a functional rhodopsin gene is puzzling.

However, the use of action spectroscopy and chromophore replacements with retinal analogs indicated that rhodopsins are the most likely photoreceptors that guide zoospore motility in the chytridiomycete fungus *A. reticulatus* (57). It is possible that rhodopsins play a key role in zoospore motility in other members of the Chytridiomycota.

BLUE-LIGHT SENSING: THE WC SYSTEM

In *N. crassa*, which is considered the model fungus for the study of blue-light responses, it has been found that most blue-light responses are controlled by WC-1 and WC-2 (128). In the dark a WCC composed of a WC-1/2 heterodimer is formed (54) that binds to light response elements in the promoters of light-responsive genes. However, this complex appears to be unable to trigger transcription *in vivo* (77, 129, 130). It is believed that upon exposure to light, structural changes in the WC-1 LOV domain take place, resulting in changes in the quaternary structure of the WCC that make it active (54). Upon activation by light, the WCC transiently binds to the light response elements of early light-responsive genes. Subsequently, transcriptional initiation takes place, which has been associated with transient phosphorylation events of WC-1 preceding removal of WC-1 from light response elements (130–133) (Fig. 3). This mechanism is assumed to be similar in many

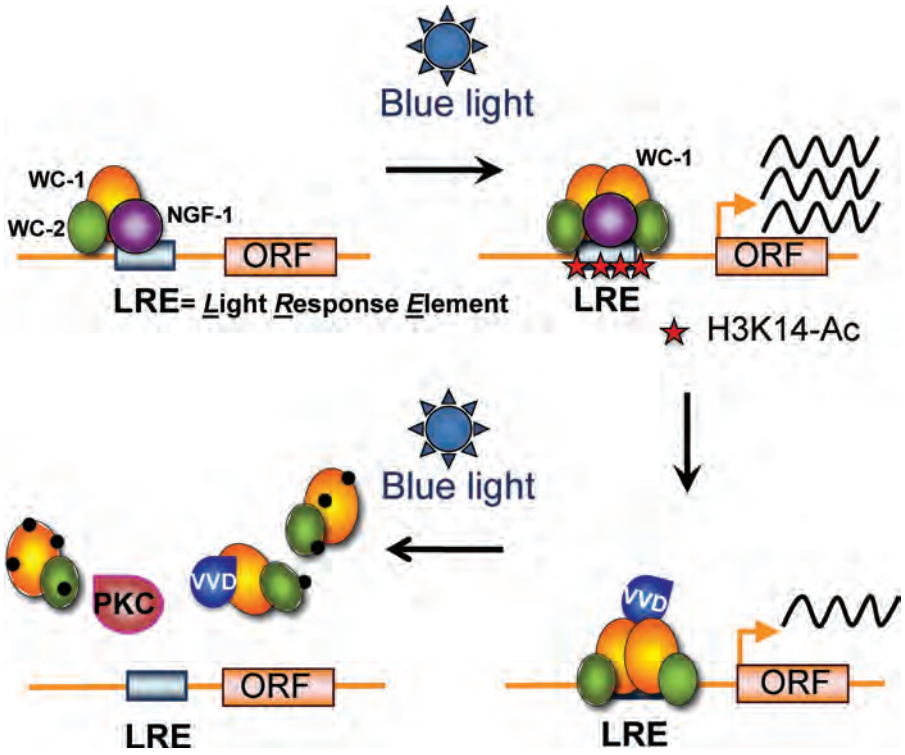


Figure 3 Model for WCC-dependent light signaling in *Neurospora crassa*. A simplified model for the activation of transcription by light and photoadaptation. Light reception by the FAD chromophore of WC-1 should trigger the formation of a flavin-cysteinyl adduct, causing a conformational change that leads to WCC dimerization, chromatin remodeling through the histone acetyltransferase NGF-1, and the activation of gene transcription. The modified histones are shown by stars at the site of promoter binding. Light exposure stimulates the transcription of *vvd*, *frq*, and other light-induced genes. Newly synthesized VVD competes with the light-activated WC-1 and disrupts the formation of WCC dimers, reducing WCC binding to the promoter. The WCC bound to VVD is not transcriptionally active, and it results in the attenuation of the response to light. Different fractions of the light-activated WCC are stabilized by FRQ (not shown) and transiently phosphorylated (black dots) and partially degraded, probably through an interaction with the protein kinase C (PKC) and other kinases and phosphates, some of them not yet identified (not shown).

other fungi that respond to light using WC orthologs, although in most cases this has not been proven (1).

Chromatin modifications are also involved in the photoinduction of gene expression regulated by the WCC (Fig. 3). Light-induced acetylation of promoter histone H3-K14 appears to be essential for the activation of light-induced genes (134, 135). Furthermore, a strain expressing a mutant allele of H3 (*hH3K14q*) that cannot be acetylated at K14 behaves like a *wc-1* mutant strain (135). Such histone acetylation is carried out by the acetyltransferase NGF-1, which directly interacts with WC-1 (134). Recently, H3K9me3 DNA methylation by the methyltransferase DIM-5 has been found to be involved in the repression of light-induced gene expression (136). Thus, there are at least two levels of control of WCC light-induced expression of genes at the chromatin level.

Organisms must be capable not only of perceiving the presence or absence of light but also of detecting subtle changes in its intensity to elicit adequate behavioral and developmental responses. Consequently, they have evolved mechanisms to adapt to prolonged exposure to light while retaining sensitivity to changes in its intensity. After prolonged exposure to light, gene expression undergoes photoadaptation in *Neurospora* and *Trichoderma*, returning to basal levels after 1 to 4 h of exposure to light (137, 138). To remain sensitive to an additional increase in ambient light intensity, both fungi are able to adapt to various levels of light intensity (137–139).

In *N. crassa*, VIVID (VVD) is a small LOV domain-containing blue-light photoreceptor that functions downstream of the WCC to negatively regulate the light responses initiated by the WCC. In light VVD forms a rapidly exchanging dimer in solution, suggesting the possibility that the LOV domain of VVD could interact with other PAS domains, including those found in the WCC proteins (84). Indeed, it was shown that there is a physical interaction between VVD and the WCC that results in a reduction of the transcriptional response, which could explain photoadaptation (87, 137, 140, 141) (Fig. 3). Further, a data-driven mathematical model proposes that VVD allows *Neurospora* to detect relative changes in light intensity. In this model VVD acts as an inhibitor of WCC-driven gene expression and as a positive regulator that sustains the responsiveness of the photosystem. The authors of that model suggest that this function is carried out by a futile cycle involving the light-induced sequestration of the active form of WCC by VVD and the replenishment of the activatable WCC pool through the decay of the photoactivated state (142).

RCO-1 and RCM-1, the *Neurospora* homologs of the corepressor complex Tup1-Ssn6 in yeast, play a role in photoadaptation. RCO-1 and RCM-1 accumulate in *Neurospora* nuclei (107) and interact to form a repressor complex similar to that observed in yeast (143). When exposed to five hours of light, the *rco-1* and *rcm-1* mutants show a sustained expression of light-induced genes, suggesting that the RCO-1/RCM-1 complex is involved in photoadaptation. The absence of the RCO-1/RCM-1 repressor complex leads to a reduction in the amount of VVD that is available for the regulation of the WCC. The reduction in the amount of VVD results in increased WCC binding to the promoters of light-regulated genes in the dark and after long exposures to light, leading to the modification of photoadaptation that has been observed in *rco-1* and *rcm-1* mutants. These results indicate that the photoadaptation phenotype of mutants in the RCO-1/RCM-1 repressor complex is, at least in part, an indirect consequence of the reduction of *vvd* transcription and the resulting modification in the regulation of transcription by the WCC (144).

Photoadaptation has been observed in other fungi too. In *P. blakesleeanus* the activation of *hspA* gene expression by light was transient, which suggested the presence of a photoadaptation mechanism similar to that described for *Neurospora* and *Trichoderma*. However, photoadaptation of *hspA* was not prevented by changes in light intensities or dark incubations, unlike photoadaptation in *Neurospora* (145). Photoadaptation has been observed for the light-dependent transcription of the developmental regulator *brlA* in *A. nidulans* (59). The differences in the sensitivity to changes in light intensities, and the absence of a *vvd* homolog in the genomes of *P. blakesleeanus* or *A. nidulans* (Fig. 2), suggests the operation of a molecular mechanisms for adaptation in these fungi that is different from that described for *N. crassa*.

In addition to the central blue-light perception system constituted by the WCC, biochemical and molecular data suggest the participation of at least two light perception systems that regulate photoconidiation in *T. atroviride* (64, 146, 147). One of the proposed signal transduction pathways participating in this alternative light perception system involves cAMP, given that protein kinase A activity increases and induction of gene expression have been observed after a pulse of blue light in Δ*blr1* mutant strains (147). Additionally, as mentioned above, members of the cryptochrome/photolyase family have functions as regulators of gene expression in several fungi (1, 19, 111, 148, 149).

RED-LIGHT SENSING AND THE INTERPLAY WITH BLUE-LIGHT AND STRESS-SENSING SYSTEMS

In *A. nidulans* light can have activating and repressing functions. Whereas asexual sporulation occurs in light, sexual development is repressed and occurs in the dark. Interestingly, both red and blue light stimulated asexual sporulation, and for full induction, both light qualities were required (34, 150). This suggested the involvement of both FphA and the blue-light-sensing WC system (LreA and LreB). However, whereas deletion of *fphA* caused a reduction of conidia production, deletion of *lreA* and *lreB* caused a slight increase in conidia formation, suggesting a positive function for FphA and a negative function for LreA (34). Red light inhibits cleistothecia formation more effectively than blue light, but the combination of the two wavelengths increases the inhibition to the level of white light. Deletion of the phytochrome gene caused a slight increase, and deletion of *lreA* caused a decrease in the number of cleistothecia. The latter effect suggests a function of LreA independent of light. To analyze the effects of the light regulators at the molecular level, light-regulated genes were identified in genome-wide analyses (59). Two of those genes, *ccgA* and *conJ*, were chosen for further analysis, because the orthologs were known to be light regulated in *N. crassa*. In contrast to the effect of light on sporulation, both genes were highly upregulated by red but not by blue light (34, 150). Because *ccgA* and *conJ* do not play any obvious role in asexual development, it is possible that the regulation of the two genes is distinct from the regulation of genes involved in conidiation. In addition, it has to be considered that sporulation or cleistothecia formation involves hundreds of differentially expressed genes. Some of these genes may be regulated like *ccgA* or *conJ*, but others may be regulated differently. Therefore, *ccgA* and *conJ* will be discussed as models to understand the effects of light at the molecular level.

Because FphA, LreA, and LreB were found within the nuclei, direct interactions of those proteins with the two promoters were feasible. Indeed, LreA was detected bound to the *ccgA* and *conJ* promoters in the dark and was released after illumination (150). Phytochrome, however, could not be detected at the promoters of these genes (Fig. 4). Thus, LreA/B could have a repressing effect and FphA an activating function without interacting directly at the promoter level, raising the question of how the information from FphA could be transmitted to the promoters. One key protein could be VeA (velvet A), another well-known fungal regulator involved in the light response (151–153). VeA contains a domain with structural similarity to NF-κB, a well-known transcription factor (154). It localizes to nuclei, and we found a physical interaction between VeA and FphA within nuclei (34). It was demonstrated that it is a multiphosphorylated protein, and a phosphorylation code has been postulated to explain the involvement of the protein in many different pathways (155, 156). The localization and accumulation of VeA in nuclei appears to be dependent on the phosphorylation of certain amino acids, where it binds to the *ccgA* and *conJ* promoters. However, this was independent of the illumination conditions, and therefore the exact role of VeA is still unclear. Apparently, VeA is required for LreA binding to the promoters (150). In summary, it appears that only FphA has an essential function in gene induction and that other proteins studied so far serve minor functions, probably allowing the integration of different regulatory cues. A breakthrough for the understanding of signal transduction from FphA to the gene expression level was achieved using a classical mutant-screening approach, where SakA, the stress-activated kinase, was identified (4). This indicates a link between light and stress signaling and a role for the transcription factor AtfA as a central component of light-dependent gene induction (157) (discussed below). In agreement with these recent findings connecting phytochrome signaling stress, mitogen-activated protein kinase (MAPK) signaling has been studied in *Beauveria bassiana*, in which phytochrome was shown to be involved in multistress tolerance (158). However, a mechanistic link between light sensing and other stresses has not yet been established in *B. bassiana*.

Besides a role of phytochrome in triggering the activity of transcription factors, a role in chromatin remodeling in *A. nidulans* is also likely (Fig. 4A). The acetylation level of the *ccgA* and *conJ* promoters changed upon illumination, and this change depended on LreA and FphA (150). It was also found that LreA interacts with the acetyltransferase GcnE and the histone deacetylase HdaA. Since FphA interacts with the WCC, it could be that FphA indirectly modulates the activity of these enzymes, but there is also some evidence that FphA directly interacts with the two enzymes (150). Hence, phytochrome-dependent gene regulation appears to occur at transcriptional and chromatin-structural levels. Unfortunately, the interplay between LreA, LreB, and phytochrome in this process is not yet well understood.

Phytochrome interacts with several other proteins, and these interactions could be indicative of its function as well. It is possible that some of the interaction partners modulate or control the phosphorylation

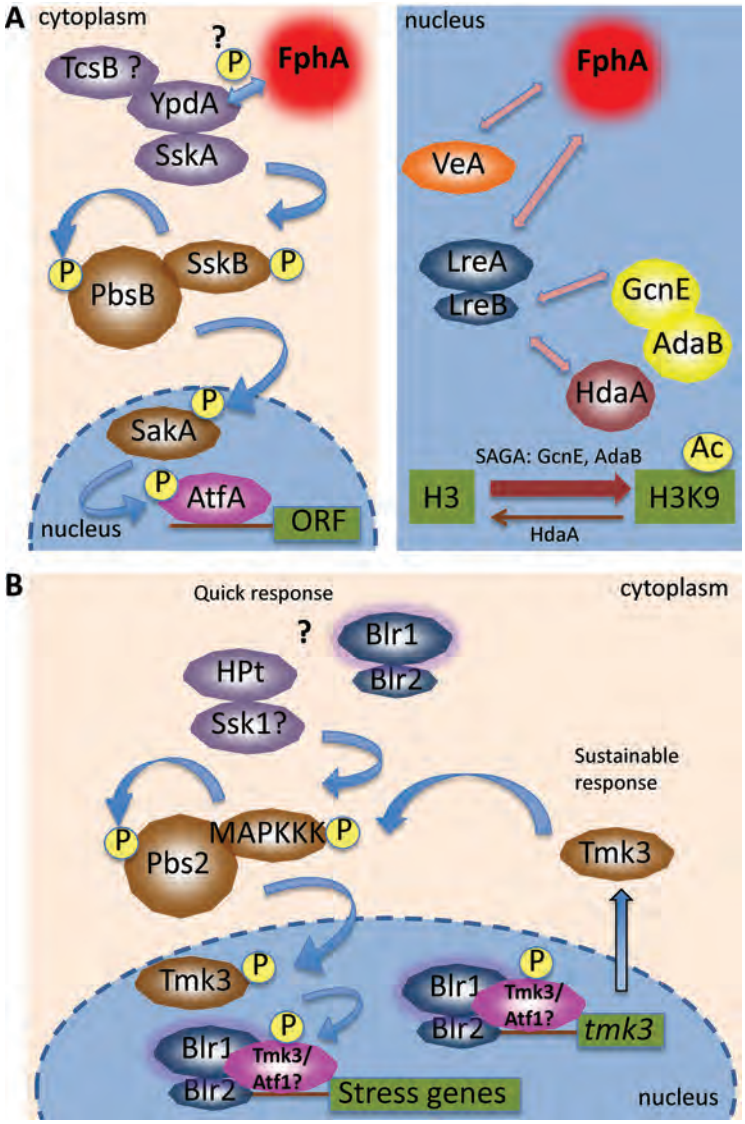


Figure 4 Phytochrome functions in light regulation in *Aspergillus nidulans* and the link of light and stress sensing in *A. nidulans* (A) and *Trichoderma atroviride* (B). (**A, left panel**) There is good evidence that the light signal is perceived by FphA in the cytoplasm and transmitted into the nucleus by activating the SakA stress signal pathway. SakA becomes phosphorylated, shuttles into the nucleus, and activates the transcription factor AtfA. (modified after 177) (**A, right panel**) Light signaling also involves chromatin remodeling of the promoters of light-regulated genes such as *ccgA* or *conJ*. It was shown that the acetylation level of lysine 9 of histone H3 increases upon illumination, that LreA interacts with the acetyltransferease GcnE and the histone deacetylase HdaA, that deletion of the SAGA/Ada complex component AdaB causes reduction, whereas deletion of *hdaA* causes induction of the photoinduction, and that changes of lysine 9 in histone H3 phenocopy the phenotypes of *adaB*- or *hdaA*-deletion strains. VeA is always bound to the *ccgA* or *conJ* promoter, whereas LreA leaves the promoter upon illumination. Hence, LreA could keep GcnE inactive and stimulate HdaA in the dark. The situation would be reversed after illumination, and the acetylation level of the lysine residue 9 of histone H3 would increase. There is evidence that GcnE is further activated through FphA. Lysine 9 acetylation was dependent on FphA, but an interaction between the two proteins was only shown by split YFP and could not be verified by Co-IP. The arrows indicate protein interactions verified by different methods. It should be noted that the current models rely solely on the results obtained with two light-regulated genes, *ccgA* and *conJ*. (**B**) The link between light and stress regulation in *T. atroviride*. In a quick response light causes phosphorylation of the MAPK Tmk3, which requires the MAPKK Pbs2. Nevertheless, it is still unclear where the WCC is linked to the Tmk3 MAPK pathway. At the promoter of a set of light-regulated genes the WCC could interact either with Tmk3 or with a not-yet-identified AtfA ortholog. Light also stimulates the transcription of the *tmk3* gene, giving rise to higher levels of Tmk3, which may aid in keeping a sustained response.

activity of FphA. Indeed, it was shown that the autophosphorylation activity increased if inactive phytochrome was added (97). New insights and hypotheses into red-light signaling in fungi may also come from the comparison with red-light signaling in plants (159).

The current model for phytochrome-dependent gene regulation in fungi mostly relies on findings in *A. nidulans*, and it will be interesting to compare the sys tem with other fungi in which phytochrome is likely to play a role. For example, conidiophore production in *B. cinerea* is induced by UV light, but conidia formation then occurs in the dark. Blue- but also red-light illumination inhibits spore production (33, 160). The molecular basis for red-light sensing in this fungus has not been investigated in detail yet, although three phytochrome-encoding genes were identified in the genome (102). In *Trichoderma*, red light provokes a reduction in mycelial growth and also has an impact on the transcriptional regulation of some genes, indicating the participation of a phytochrome in these responses (45, 161). However, expression of the set of genes that respond to red light is also controlled by blue light (64). These observations suggest that blue and red light photoreceptors act together to regulate expression of these genes. In this regard, it has been suggested that in *N. crassa* the photoreceptors CRY-1, NOP-1, and PHY-2 modulate the activity of the WCC, presumably through the light-dependent activation of a putative repressor of the WCC (162). Furthermore, expression analysis of a small set of genes in the Δ*blr1*, Δ*phr1*, and Δ*cry1* mutants of *T. atroviride* showed that the three potential blue-light photoreceptors are involved in the control of gene expression in both blue and red light (64).

LIGHT SIGNALING TO STRESS MAPKs

Most of recent research in fungal photobiology has focused on the identification and characterization of the WC photoreceptor complex and other photoreceptors, but little attention has been paid to the components of the signal transduction pathway that relies on the light input into the cell. The key role of the transcription factor and blue-light WC photoreceptor complex in *N. crassa* and other fungi suggests a prominent role for light-dependent gene regulation in fungal photobiology. However, it is possible that the WCC and other photoreceptors regulate other aspects of fungal biology at the posttranscriptional level through the action of a signal transduction pathway that starts with the reception of light by the photoreceptor. As mentioned earlier, a connection between light sensing and MAPK signaling has been established in some fungi, which could constitute a more general aspect of fungal photobiology.

Indeed, many environmental signals are transmitted through conserved three-tiered phosphorylation cascades composed by a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). Active MAPKs phosphorylate multiple targets including other enzymes and usually translocate from the cytoplasm to the nucleus to phosphorylate nuclear targets such as transcription factors. Stress-activated protein kinases (SAPKs) are MAPKs that are specialized in transducing stress signals. In fungi, SAPK input involves the participation of phosphorelay signal transduction systems composed by different sensor histidine kinases (HK), a phosphotransfer protein (HPt) Ypd1, and a response regulator (163) (RR) (Fig. 4).

The first report connecting light to MAPK signaling in fungi showed a circadian rhythmic activation of the SAPK OS-2 in *N. crassa*, which was dependent on WC-1, the clock component FRQ-1, and the RR RRG-1 (164). Notably, OS-2 phosphorylation induced by osmotic stress required RRG-1 but not the WC-1/FRQ-1 oscillator, indicating that OS-2 regulation by osmotic shock and circadian clock occurs through different pathways, upstream of RRG-1. Later it was shown that WC-1 mediates SAPK light input by binding to the promoter of the *os-4* gene, encoding MAPKKK OS-4, both in response to light and in a rhythmic fashion under constant darkness. Deletion of *os-4* WC-1 binding sites disrupts *os-4* mRNA and OS-2 phosphorylation rhythmic oscillations. Since WC-1 is also indirectly required for antiphase rhythmic expression of the HPt gene *hpt-1*, it was proposed that such WC-1-mediated antiphase expression of positive (OS-4) and negative (HPT-1) SAPK regulators is coordinated to enhance the rhythmic activation of the OS-2 pathway (5). The negative relationship between phosphotransfer HPt proteins and SAPKs has been indicated by the fact that the elimination of HPt proteins is lethal in wild-type backgrounds in *A. nidulans* (165) and *N. crassa* but is viable in an *os-2* null background (166). Furthermore, the transcription factor ALS-1, also reported as ATF-1 and proposed to act downstream of OS-2 (167), is required for the circadian rhythmic oscillation of OS-2 responsive genes *bli-3*, *ccg-1*, *cat-1*, *gcy-1*, and *gcy-3*, in the absence of osmotic stress (168).

More recently, it has been shown that transcript levels of the *T. atroviride tmk3* gene, which encodes SAPK Tmk3, are increased after blue-light exposure, and this depends on the WC homolog Blr1. Likewise, light-induced conidiation and the induction of the

blue-light-regulated genes *blu1* and *grg2* is drastically reduced in mutants lacking Tmk3 or the upstream MAPKK Pbs2 (3). Although it is not known if transcription factor Atf1 is required for light induction of these genes, these data show that through Blr1, light regulates SAPK signaling at the transcription or mRNA stability levels. Because the *tmk3* promoter contains putative Blr2-binding sites, the authors propose a model in which the Blr1/Blr2 photoreceptor complex could regulate *tmk3* expression and also mediate light input to the SAPK pathway upstream from Pbs2 and/or interact with Tmk3 in the nucleus to promote the transcription of stress and light-responsive genes (Fig. 4, B). It seems possible that Blr1/Blr2 could also regulate the expression of MAPKKK and Hpt1 proteins, as in *N. crassa* (5). In addition, Blr1 is required for Tmk3 transient phosphorylation after mycelia exposure to a light pulse or constant illumination. One possibility is that *T. atroviride* Blr1/Blr2 forms a complex with the HK equivalent to phytochrome FphA, as it occurs in *A. nidulans* (34, 155), and transduces direct phosphorylation signals to the SAPK pathway (4). Alternatively, Blr1/Blr2 could feed the pathway downstream of Tmk3. As in *S. pombe* (169) and *A. nidulans* (170), Tmk3 is required for resistance to different types of stress (3), and SAPK activation by heat shock (171) and nutrient limitation (172) in *S. pombe* occurs downstream of Spc1/Sty1 through inhibition of specific tyrosine phosphatases.

As mentioned before, red-light sensing has recently been connected to MAPK signaling in *A. nidulans* through the phytochrome FphA (4), which is a hybrid HK (34, 155). This fungus contains 15 HKs, and the function of most of them is unknown. Genetic evidence indicates that the HK NikA transmits osmostress and fungicide signals to the phosphotransfer protein YpdA and to the response regulator SrkA, which is coupled to the SAPK SakA (170), as well as to the SAPK-independent RR called SrrA (165). SakA was also identified as HogA and shown to be involved in gene regulation in response to osmotic stress (173). Upstream, MAPKK PbsB and MAPKKK SskB regulate SakA (174), which is phosphorylated in response to multiple types of stress, including osmotic, oxidative (170), nutrient starvation (157), and hypoxia (Sánchez and Aguirre, unpublished). Stress-activated SakA translocates to the nucleus, where it interacts with transcription factor AtfA, which is required for induction of several genes, including catalase genes *catA* and *catB*, and both Δ*sakA* and Δ*atfA* mutants are sensitive to oxidative stress (157). Both SakA and AtfA are also required for osmotic-induced gene expression (175). It is worth mentioning that SakA also interacts with MAPK-activated protein kinase SrkA and that both show H_2O_2-induced interaction with other proteins involved in SAPK signaling, cell-cycle regulation, DNA damage response, and mitochondrial function (176), because such interactions might become relevant to the light-sensing process. In addition, SakA and AtfA play important roles during development, repressing sexual development and being activated during asexual development (170). Δ*sakA* intact conidia progressively lose their viability, and this is consistent with the fact that SakA accumulates in asexual spores (conidia) in an AtfA-dependent manner. SakA becomes phosphorylated during conidia development and needs to be dephosphorylated for germination to take place, indicating that this SAPK plays essential roles in the transition between growth and differentiation (157).

Yu et al. isolated "blind" mutants by using a light-responsive promoter fused to a nutritional marker (4). By genomic sequencing, these authors found that different mutants carried inactivating mutations at the MAPKKK (SskB), MAPKK (PbsB), or MAPK (SakA) components of the SAPK pathway. Indeed, several lines of evidence support the involvement of this pathway in red-light and, to a minor extent, blue-light sensing. First, blind and Δ*sakA* mutants show the same phenotypes in terms of asexual/sexual development. Second, deletion of FphA upstream (SskA, SskB, PbsB) or downstream (AtfA) SAPK components results in failure to induce *ccgA* and *conJ* genes in response to light. Third, FphA physically interacts with the HPt protein YpdA, as shown by BiFC and coimmunoprecipitation. Fourth, light induces increased SakA phosphorylation and its nuclear localization in a way that depends on FphA but is independent of blue-light receptors LreA and LreB. Somewhat unexpectedly, blue light partially induces SakA nuclear localization, not as strongly as red light, and this response depends only on FphA. Notably, FphA-mediated regulation of SakA is not required for osmotic stress-induced activation of SakA.

Under certain conditions, FphA histidine kinase is able to autophosphorylate and transfer the phosphoryl group to the response regulator of a second interacting FphA molecule *in vitro* (97). Both phosphorylation sites in FphA, the histidine in the histidine-kinase domain and the aspartate in the response regulator domain, are essential for function (150). *In vitro* experiments showed that FphA autophosphorylation activity was higher in light than in dark (97), and the phosphotransfer from YpdA to FphA has been shown *in vitro*, using a truncated version of FphA lacking the first 677 amino acids (101). Taken together,

these results clearly support the role of FphA as a light-sensitive histidine kinase connected to the SakA-AtfA pathway at the YpdA level. However, they tell little about the actual phosphotransfer dynamics *in vivo*. Nevertheless, it is clear that *in vivo* red-light illumination results in the activation of the MAPK SakA and the regulation of red-light-responsive genes through the AtfA transcription factor.

Discussing these results, Yu et al. considered that FphA had a higher kinase activity in the dark, when actually it shows red-light-induced autophosphorylation *in vitro* (97). This led to a proposed model in which under dark conditions the HK FphA phosphorylates and associates with the HPt protein YpdA, and this leads to phosphorylation of the response regulator SskA and inactivation of SakA. Light would cause YpdA dephosphorylation and activation of the SakA pathway (4, 177). Such a model is consistent with the fact that light inhibits the germination of conidia in an FphA-dependent way (42), because it is known that high levels of phosphorylated SakA prevent spore germination (157). However, the exact mechanism by which red light activates SakA remains to be demonstrated.

LIGHT: A STRESS, A SIGNAL, OR BOTH?

The presence and functioning of different photoreceptors in fungal cells and the low threshold for some responses to light suggests that light is used as a source of environmental information, because it clearly occurs during the phototrophic reaction of Phycomyces. The connection between light, photoreceptors, and the stress MAPK can be interpreted as a mechanism that uses the light cue to activate the SAPK pathway to anticipate and prepare the cell for later stress (164). In this scenario, light is a reliable signal about incoming changes in temperature, osmotic stress, oxidative stress, and damaging UV radiation. Such alarm signaling can occur many times during the day as hyphae grow in and out of their substrate or in circadian rhythms as an alarm system that every morning prepares fungi for the stress of the day. However, recent evidence suggests that excessive light can also directly cause cell stress (i.e., the production of H_2O_2 and other reactive oxygen species [ROS]), which in turn might activate photoreceptors and SAPKs. The fact that the replacement of key cysteine 196 by threonine in *T. reesei* ENV1 (138), a homolog of *N. crassa* VVD, abolishes adaptive responses to both light and oxidative stress *in vivo* (85) suggests that light and ROS signals perceived by a single protein could be transmitted to SAPK or other signal transduction pathways.

Additional, less direct evidence connecting light irradiation and ROS production has been reported in other fungi. In *B. cinerea*, constant light impairs growth of a wild-type strain, and this is more drastic in mutants lacking the WC homolog BcWCL1. Notably, this can be enhanced and reversed by adding H_2O_2 and antioxidants, respectively (102). In *N. crassa*, deletion of the superoxide dismutase *sod-1* gene results in circadian rhythmic conidiation (178). Microarray analyses of the light response of *A. nidulans* and *N. crassa* revealed that stress-related genes are activated by light (59, 137). Similarly, transcriptome analyses of this response in *T. atroviride* showed that a significant proportion of the light-induced genes are related to oxidative and other types of stress responses (64). Furthermore, a recent proteome analysis of the *Penicillium verrucosum* response to light indicated that many of the induced proteins are involved in responses to stress (179). In *A. fumigatus* exposure to light results in enhanced resistance to acute UV and oxidative stresses and an increased susceptibility to cell wall perturbation (41), and in *B. bassiana* the putative phytochrome (*Bbphy*) is not only a photoreceptor essential for asexual development, but it also acts as a regulator of the fungal responses to a variety of stresses, including oxidative stress (158).

Evidence linking light and ROS production has also been reported in animal systems. In zebra fish light induces H_2O_2 production, while external H_2O_2 induces cryptochrome *zCry1a* and period *zPer2* gene expression and the subsequent circadian oscillation of *zPer1*. Notably, the expression of the catalase gene *zCat* shows antiphasic oscillation to *zCry1a* and *zPer2*, and consistent with this, *zCat* overexpression causes reduced induction of *zCry1a* and *zPer2* by light (180).

All these results clearly show a connection between light, circadian rhythms, and redox metabolism, which is consistent with the idea that monitoring ROS levels is essential in triggering cell differentiation (181). Such interesting relationships can now be studied from the perspective of the established connections between light, stress, and SAPK signaling.

WHY SO MANY PHOTORECEPTORS?

Although the effect of light on fungal development and physiology was recognized long ago, the molecular analysis reveals a complex picture of light sensing. It appears that light is an important environmental cue mainly to adapt fungal biology to stressful or harmful conditions. One of the open questions, however, is why several photoreceptor and light-sensing systems were established during evolution if their role was only to

detect light or darkness. Although normally one photoreceptor system appears to dominate the light response in a given species, such as the blue-light-sensing system in *N. crassa* or the red-light response in *A. nidulans*, there is very good evidence that the additional systems also play certain roles. It could be that the different photosensing systems evolved for the sake of robustness in light sensing. However, one system does not seem to substitute for the lack of the other, suggesting that sensing different light qualities provides an advantage. Although monochromatic light is rare in nature, the composition of daylight may vary significantly. For instance, blue light penetrates deeper into water than red light, red light is more present in the evening, plants absorb the blue and red range of the spectrum, and fungi living in the shade of plants are exposed to more green light. Fungi are able to sense these spectral differences as additional environmental cues to adapt to their lifestyles accordingly. The existence of several photoreceptors and their relative conservation during evolution also suggests that the light stimulus needs to be transduced to different biological processes, which might be more difficult with a single photoreceptor. In *B. bassiana* phytochrome appears to be also involved in sensing the length of the illumination period. Whereas a wild-type strain grown for 7 days produced the maximum amount of conidiospores with 3 h of white light illumination (21 h darkness), the phytochrome mutant required 16 h of light (8 h darkness) (158). Although such adaptive changes may be rather subtle in other fungi and hard to detect under laboratory conditions, they might be important for fitness and survival in nature.

In addition to our improved understanding of photoreceptors in light responses, it appears that some of these proteins also serve functions in the dark. One prominent example is the WCC in *N. crassa*. This is the positive element in the circadian clock system, and it enables its entrainment by light. However, one of the conspicuous features of the clock is its free running in the absence of external stimuli (53). As germination is delayed in the dark in the absence of phytochrome in *A. nidulans* (42), phytochrome also appears to have functions in the dark. A still open question is, Which was the ancestral function of photoreceptor proteins? We think that initially, fungal vision originated as a mechanism to anticipate the stress and damage caused by light. Alternatively, it is possible that the fungal ancestor, being a motile cell living in the ocean, could use low and different colored light as a guide, explaining the large number of different photoreceptors in all fungi. Once fungal ancestors changed their living habitat to soil and plant surfaces, the primordial light detection system was adapted as a protective system to predict excess light.

CONCLUDING REMARKS

Fungal genome projects have allowed the identification and characterization of several photoreceptor genes, many of them unexpected. The identification of the WCC in *N. crassa* has served as a model for other fungi, and in most cases mutants in the homologs of the WCC result in blind phenotypes. On the other hand, *A. nidulans* displays a pronounced red-light response, and phytochrome has been characterized in some detail. Recently, also in *N. crassa*, a role for phytochrome was assigned. In addition, opsin may be a new player in photosensing. When all this is taken together, it becomes evident that fungi employ several photoreceptors, and we are just at the beginning of unraveling their functions and interlinked signaling cascades. Given the broad impact of light on fungal growth, development, and physiology, the understanding of the roles of and interactions among fungal photoreceptors will be a major avenue of fungal research in the near future.

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Stress Adaptation

21

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INTRODUCTION

Planet Earth plays host to an extravagantly diverse range of fungal species. Recent estimates suggest the probable existence of as many as 3 million fungal species (1), and the *circa* 75,000 of these that have been characterized to date display a wide range of lifestyles. Many fungi occupy specific niches within natural environments, playing essential roles in nutrient scavenging and recycling. Some thrive in close harmony with species from other kingdoms, a superb example being the mycorrhizal fungi, which display mutualistic interactions with plants. Other fungi are pathogenic, causing devastating infections of plants or animals. Indeed, the global threats that fungi pose to human health and food security are being increasingly recognized (2). Fortunately, a relatively small number of fungal species cause infections in humans (circa 400 species are described in the *Atlas of Clinical Fungi* [3]). Some of these fungi normally occupy environmental niches but are capable of colonizing and damaging human (or animal) tissues, whereas other fungi appear to be obligately associated with their host.

These diverse fungal niches are dynamic in that they display fluctuations in local parameters such as temperature, water balance, pH, or the levels of particular compounds such as nutrients and reactive oxygen and nitrogen species. These fluctuations are often capable of perturbing cellular homeostasis and causing molecular damage, thereby imposing stress on the fungal cell. Consequently, fungal cells must be able to adapt to these dynamic changes if they are to survive, grow, and colonize any niche. This stress adaptation is dependent on three fundamental principles. The first is the ability to detect environmental signals, i.e., the changing inputs from the local environment. The second is the ability to transduce these signals to regulate the cellular processes that mediate the stress adaptation. The third represents the adaptive responses themselves that allow cells to survive the stress. These adaptive processes either counteract or detoxify the initial stress and repair or remove the molecular damage caused by that stress. These fundamental principles must apply to all fungi.

Given the elemental nature of environmental stresses, it is not surprising that there are fundamental similarities across the fungal kingdom (and beyond) with regard to the basic cellular processes that mediate adaptation to specific stresses. For example, evolutionarily divergent ascomycetes and basidiomycetes induce protein refolding mechanisms in response to changes in temperature (4, 5) and the synthesis of antioxidants following exposure to oxidative stresses (6, 7). However, different niches exert different evolutionary pressures, and this has led to considerable diversity between fungal species with regard to the robustness of specific stress responses. For example, the yeast *Debaryomyces hansenii*, which is found in hypersaline waters, can tolerate higher levels of salt than *Saccharomyces cerevisiae* (8), which seems to have evolved to grow on fruit and to be disseminated by wasps (9). Also, *Candida glabrata*, which is a fungal pathogen of humans that is relatively resistant to phagocytic killing (10), displays extremely high levels of oxidative stress resistance compared to other yeasts (11). This evolutionary tuning of stress resistance to the local niche has led to some divergence between fungal species in the regulation of the cellular processes that mediate adaptation to some stresses.

This chapter summarizes our current understanding of the mechanisms underlying fungal stress adaptation and the regulation of these responses. We focus on those

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stresses that have been perceived to be the most relevant and hence have been most studied to date and the experimentally tractable model fungi in which stress adaptation mechanisms are best characterized. Also, we compare and contrast these outlooks on stress sensing to those from other fungi which have provided fascinating insights into niche-dependent stress adaptation.

ADAPTING TO INDIVIDUAL STRESSES

Heat Shock

Temperature modulates diverse facets of biology and disease (Fig. 1). Organisms across the tree of life must contend with changes in temperature that can manifest across a multitude of scales, from global climate change, to seasonal environmental change, to abrupt change associated with transitions in environmental niches. For microbial pathogens, temperature can signal the successful infection of a host and serves as a central cue governing proliferation, developmental programs, and virulence (12). As an example, fever is a ubiquitous response to infection, with elevated febrile temperatures thought to serve as an adaptive host response to restrict microbial proliferation. In a broader context, mammalian endothermy may have evolved as a strategy to minimize infections caused by fungal species, most of which have a diminished capacity to proliferate at elevated temperatures (13). Of the ~3 million fungal species estimated to exist, less than 0.1% are able to cause disease in mammals, and this can largely be attributed to most rapidly losing their capacity for growth above ambient temperature (14).

Thermal transitions have a profound impact on fungal development and virulence. For example, virulence of the dimorphic fungal pathogens is controlled by a temperature-dependent change in morphology (15). *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum* are key species that exemplify the characteristic response to temperature of the dimorphic fungi: these species grow as filamentous molds in the soil in response to ambient temperature and convert to growth as yeast cells in response to host temperature upon inhalation of spores into mammalian lungs. The polymorphic fungus *Candida albicans* is another fungal pathogen for which temperature induces a dramatic morphological change (16). In contrast to the dimorphic fungi, *C. albicans* proliferates in the yeast form at ambient temperatures, and elevated temperature promotes a transition to filamentous growth. A temperature of 37°C is required to enable filamentation in response to diverse cues such as serum, and a further increase in temperature to 39°C is sufficient to induce filamentation in the absence of other cues (17). Beyond morphogenesis, temperature exerts a profound influence on diverse aspects of *C. albicans* biology, including mating, phenotypic switching, and drug resistance (17). In addition to the phenotypic consequences of growth at sustained elevated temperatures, fungi also have a profound response to acute but temporary increases in temperature that is referred to as a heat shock response. Strikingly, a transient heat shock can activate

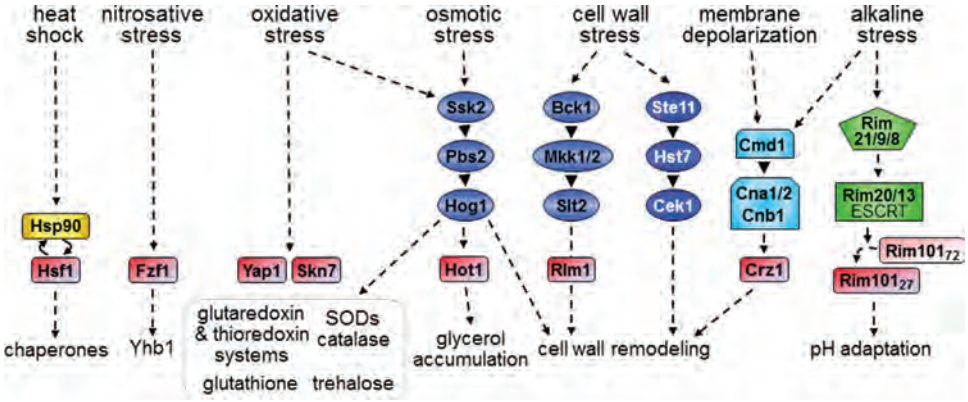


Figure 1 Cartoon summarizing stress pathways in the model fungus *S. cerevisiae*. See text. This figure summarizes some, but not all, of the known components of these signaling pathways. Components of MAPK signaling modules are highlighted in blue, transcription factors in pink, components of the calmodulin-calcineurin pathway in cyan, Rim pathway components in green, and the molecular chaperone Hsp90 in yellow. Note that the *C. albicans* Cek1 MAPK pathway, which contributes to cell wall remodeling in this fungus, is included (dark blue ovals with white lettering).

a *C. albicans* transcriptional program that is associated with increased host cell adhesion, host cell damage, and virulence (18).

Fungi have evolved complex molecular machinery and regulatory circuits to respond to the stress induced by sustained or transient responses to elevated temperature, with the heat shock response being one of the most evolutionarily conserved stress responses in nature. Core to the heat shock response is a global arrest of translation elongation and transcriptional activation of genes encoding heat shock proteins, which include molecular chaperones that promote folding and refolding (19). In *C. albicans* and the model yeast *S. cerevisiae*, ~10 to 20% of genes in the genome are induced in response to heat shock (20, 21). This transcriptional response is orchestrated in large part by the heat shock transcription factor Hsf1. In *C. albicans*, Hsf1 binds to 49 targets constitutively and an additional 55 targets in response to heat shock, with targets enriched for functions in protein folding and entry into the host (18). Hsf1 typically binds at nucleosome-depleted regions in the promoters of target genes and recognizes three motifs with distinct binding affinities (18). Hsf1 is activated by phosphorylation, and this activation is required for virulence in *C. albicans* (19, 21).

Complex functional relationships influence mobilization of cellular responses to thermal stress. Hsf1 promotes the basal expression and thermal induction of genes encoding the molecular chaperone Hsp90 (Fig. 1). Hsp90 in turn physically interacts with Hsf1, thereby exerting a repressive effect on activation of the heat shock response (19). As a consequence, perturbation of Hsp90 function by small molecules, mutations, or elevated temperature causes activation of Hsf1 and induction of the heat shock response in the absence of thermal stress. In contrast, Hsp90 is required to mobilize a rapid transcriptional response to thermal stress, such that depletion of Hsp90 causes delayed induction of the transcriptional program induced by heat shock (18). Hsp90 influences transcriptional programs not only via effects on Hsf1, but also by modulating chromatin remodeling, nucleosome removal, and RNA polymerase II stalling (22–24).

As a molecular chaperone, Hsp90 regulates the stability and function of diverse client proteins, which include many core cellular regulators beyond Hsf1. For many client proteins, Hsp90 stabilizes otherwise metastable regulators, thereby enabling their activation in response to stress or other cues (25, 26). In the context of thermal adaptation, Hsp90 stabilizes the Hog1 stress-activated protein kinase, as well as the mitogen-activated protein kinases Mkc1 and Cek1 (27–29). In this context, Hsp90 enables signaling required for cell wall remodeling and adaptation to heat shock. Classical genetic screens and chemical genomic approaches to identify mutants that are hypersensitive to Hsp90 inhibition under distinct stress conditions have provided powerful strategies to identify novel Hsp90 client proteins and key regulators of thermal adaptation (27, 29, 30).

The circuitry underpinning thermal adaptation can be activated by a remarkable diversity of temperature-sensing mechanisms. For example, Hsp90 function is exquisitely sensitive to elevated temperature because the global problems in protein folding that arise for thermal stress create an elevated cellular demand for Hsp90 that exceeds its functional capacity to engage with client proteins (30). In the broader context, DNA, RNA, proteins, and lipids can all serve as thermosensors that sense changes in temperature to initiate crucial cellular responses and developmental programs (12).

Osmotic Stress

Managing changes in water balance is another fundamental challenge for fungi in most environments. The classic experimental model for this has been the imposition of hyperosmotic shock through addition of sorbitol or salts such as NaCl (31). This results in a sudden decrease in the intracellular turgor pressure that is required for fungal growth. The fungus must restore its turgor pressure before it can resume growth, and to achieve this, it activates the synthesis and accumulation of intracellular osmolytes such as glycerol (Fig. 1).

S. cerevisiae responds to osmotic stress by increasing the flux from glycolysis to glycerol. This is achieved by inducing the genes encoding glycerol-3-phosphate dehydrogenase (*GPD1*) and glycerol-3-phosphate phosphatase (*GPP1*). *S. cerevisiae* can also assimilate glycerol from the growth medium, although this uptake is repressed by glucose (32), which is in contrast to the response of some other yeasts (33, 34). However, the intracellular accumulation of glycerol is also dependent on restricting glycerol efflux from the cell through the plasma membrane-based aquaglyceroporin, Fps1 (35). Following the imposition of a hyperosmotic stress, the intracellular accumulation of glycerol and the restoration of turgor pressure take about 30 minutes (36), after which growth may resume.

This type of response, which involves signaling, gene regulation, and subsequent changes in metabolism, is too slow to protect the fungal cell against hypo-osmotic shock. This stress causes the immediate accumulation of water and rapid increases in cell volume which, if not countered quickly, would cause a yeast cell to burst

(37). In *S. cerevisiae*, this rapid rise in turgor pressure is alleviated by swift opening of the Fps1 aquaglyceroporin, the activity of which is regulated by protein phosphorylation. Mutations that inactivate Fps1, and hence block rapid glycerol efflux, confer hypo-osmotic stress sensitivity on the yeast cell (38). Clearly, responses to hyper- and hypo-osmotic shocks occur over differing timescales that reflect the relative imminence of potentially irreversible damage to the fungal cell.

Regarding the regulation of osmotic stress responses, the activation of Hog1 in *S. cerevisiae* has formed the paradigm of osmotic stress activation of fungal stress-activated protein kinases (SAPKs) (39). Like all mitogen-activated protein kinase (MAPK) modules, the Hog1 MAPK module comprises three tiers of kinases; the MAPKKK(s) at the top of the pathway phosphorylates and activates a downstream MAPKK, which then phosphorylates and activates the terminal MAPK. The central importance of this MAPK module in fungal stress sensing is illustrated by its involvement in diverse environmental responses in diverse fungi, which even include light sensing in *Aspergillus nidulans* (40). In *S. cerevisiae*, two functionally redundant pathways converge at the MAPKK Pbs2 to relay osmotic stress signals to Hog1. These are the Sln1 two-component signaling pathway and a pathway that contains the SH3-domain-containing Sho1 transmembrane protein. In the first pathway, loss of turgor pressure, induced by high osmolarity, inactivates the transmembrane histidine kinase Sln1 and thus halts phosphorelay through the phosphorelay protein Ypd1, leading to a rapid dephosphorylation of the Ssk1 response regulator (41). Dephosphorylated Ssk1 activates the MAPKKKs Ssk2/22 in a two-step mechanism (42), leading to phosphorylation and activation of the Pbs2 MAPKK. In the second pathway, the Ste11 MAPKKK phosphorylates Pbs2 when stimulated by osmotic stress signals received from the Sho1 branch of the Hog1 pathway (43). Many proteins are involved in the osmotic stress signaling from Sho1 to Ste11-Pbs2, including Cdc42, Ste20, Cla4, Ste50, and Opy2 (43, 44). Sho1 was originally thought to function as the osmosensor at the top of the pathway (45). However, two functionally redundant osmosensensors have since been identified: the mucins, Msb2 and Hkr1 (46). As different signaling mechanisms are employed by Msb2 and Hkr2 (47, 48), the Sho1 branch is now divided into the Hkr1 and Msb2 subbranches. While Msb2 and other Sho1 branch components also participate in the filamentous growth MAPK pathway, Hkr1 plays a specific role in Hog1 signaling (49), and this is mediated through the newly discovered scaffold protein Ahk1 (50).

Because the SAPKs are among the most evolutionarily conserved stress signaling proteins in fungi (11), it is remarkable that the mechanisms underlying the osmotic stress regulation of *S. cerevisiae* Hog1 have significantly diverged. For example, in the distantly related model yeast *Schizosaccharomyces pombe*, two-component signaling functions to relay H_2O_2, but not osmotic, stress signals to the Sty1 SAPK (51), and an orthologue of Sho1 is seemingly absent from the fission yeast genome (11). In *C. albicans*, although Sho1 pathway components have been identified and characterized, they are not required for the relay of osmotic stress signals to Hog1 (52, 53). Consistent with this, Hog1 in *C. albicans* is regulated by a single MAKKK, Ssk2, with the Ste11 MAPKKK—predicted to function downstream of Sho1—having no obvious role (54). In contrast, the available evidence does support the involvement of two-component signaling in the activation of *C. albicans* Hog1 by osmotic stress, because close homologues of the Sln1-Ypd1-Ssk1 pathway are present in *C. albicans*, and Hog1 is hyperactivated in cells lacking Sln1 (53). However, the observation that osmotic stress-induced Hog1 activation is not notably impaired in cells lacking the Ssk1 response regulator (55) suggests the presence of a novel osmotic stress-sensing pathway in this fungal pathogen. In certain *C. glabrata* isolates, only the Sho1 branch functions to relay osmotic stress signals to Hog1. This is due to a truncated *ssk2* allele which prevents signaling through the Sln1 branch (56). Intriguingly, gain of function mutations have been identified in the related Ssk2 MAPKKK in *Cryptococcus neoformans* (57), which are responsible for high basal levels of Hog1 activation in serotype A strains (58).

Hog1 has been shown to play a central role in the regulation of osmoadaptation in *S. cerevisiae*. This MAPK regulates accumulation of glycerol via transcriptional activation of *GPD1* and *GPP1* in response to osmotic stress via the transcription factors Hot1, Msn2, and Msn4 (59) and by controlling the activity of the Fps1 acquaglyceroporin (60). It should be noted that additional TORC2/Ypk1-dependent signaling mechanisms do contribute to the regulation of Fps1 and hence to survival in the face of hyperosmotic stress (38). However, Hog1 also mediates the transient delay in cell cycle progression following hyperosmotic shock by phosphorylation of Sic1 and Hsl1, and by downregulating G1 and G2 cyclins (61). Once osmo-adaptation is achieved, the yeast cell has essentially achieved a new homeostatic state in which turgor pressure has been restored in the face of the external osmotic conditions (62). Consequently, the input signal has been

dampened, Hog1 becomes deactivated, the block to cell cycle progression is released, and growth can resume.

Oxidative Stress

Reactive oxygen species (ROS) are highly damaging, reduced forms of oxygen, which include the superoxide anion O_2·, hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH). These reactive molecules damage proteins, DNA, and lipids and can trigger programmed cell death (63). All fungi that grow aerobically are exposed to superoxide anions generated as a by-product of aerobic respiration in the mitochondria (64). Environmental fungi are also exposed to ROS generated following exposure to UV light or to drugs/xenobiotics found in the environment (63). In addition, pathogenic fungi are exposed to superoxide and hydrogen peroxide ROS, which are generated by plant (65) or animal (66) host defense systems as a major antimicrobial defense mechanism. Significantly, other toxic chemicals are subsequently derived from the host-generated ROS (66).

Oxidative stress occurs when the levels of ROS exceed the antioxidant capacity of the cell which functions to maintain the intracellular redox environment in a reduced state. In response to oxidative stress, fungal cells mount a wide range of defense and repair strategies. One well-characterized and conserved response involves the rapid induction of mRNAs that encode oxidative stress detoxification and repair proteins (Fig. 1). Transcript profiling studies indicate that a set of core antioxidant genes are induced in fungi following exposure to H_2O_2 (20, 67–71). These include catalase (*CAT1*), glutathione peroxidase (*GPX*), and superoxide dismutase (*SOD*) antioxidant encoding genes, in addition to genes encoding components of the glutathione/glutaredoxin (*GSH1*, *TTR1*) and thioredoxin (*TSA1*, *TRX1*, *TRR1*) systems. An additional and very rapid response to oxidative stress, which precedes transcriptional activation, is the dynamic redirection of the metabolic flux from glycolysis to the pentose phosphate pathway. This metabolic switch is triggered by the oxidation and inactivation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, which functions to promote the generation of reducing power in the form of NADPH (72). Yeast cells also transiently delay cell cycle progression following exposure to ROS to allow DNA damage repair. For example, H_2O_2 causes a G2 cell cycle arrest in *S. cerevisiae* by activating the Rad53 DNA damage checkpoint (73). For more details on activation of DNA damage checkpoints in *S. cerevisiae*, readers are directed to the recent excellent review in reference 74. In the pathogenic fungus *C. albicans*, exposure to H_2O_2 also triggers Rad53-mediated cell cycle arrest. Interestingly, in this fungus, such genotoxic-induced cell cycle arrest promotes the formation of a filamentous hyperpolarized bud growth form (75, 76).

Orthologues of transcription factors that are vital for oxidative stress-responsive gene induction in *S. cerevisiae*, the AP-1-like bZip factor Yap1 and the Skn7 response regulator, have now been studied in many fungi (66). The elegant mechanism underlying activation of *S. cerevisiae* Yap1 is well characterized. Following exposure to H_2O_2, specific cysteine residues located within two distinct cysteine-rich domains become rapidly oxidized (77). This oxidation event, which requires the thiol peroxidase Gpx3 (78) and the Yap1 binding protein Ybp1 (79), triggers a conformational change within Yap1 that masks a nuclear export sequence (80). Consequently, Yap1 accumulates in the nucleus, resulting in the induction of Yap1-dependent genes (78). In the human fungal pathogen *C. albicans*, Cap1 oxidation is similarly regulated by Gpx3 and Ybp1 (81). However, in the model yeast *S. pombe*, Pap1 oxidation depends instead on the 2-Cys peroxiredoxin Tpx1 (82), and in the fungal symbiont *Epichloe festucae*, redox regulation of the analogous YapA factor is independent of both Gpx3 and Tpx1 (83). Thus, multiple mechanisms may exist to regulate the oxidation of fungal AP-1-like factors. Interestingly, AP-1-like factors have been found to be dispensable for the virulence of the human fungal pathogens *Aspergillus fumigatus* (84), *C. neoformans* (85), and *C. albicans* (81, 86) but are required for the virulence of a number of plant pathogens (87–89).

In *S. cerevisiae*, Yap1 collaborates with Skn7 to regulate many oxidative stress-response genes, and this may be conserved, because similar findings have been reported in *C. glabrata* (90) and *S. pombe* (91). Little is known about Skn7 activation following oxidative stress, but a study has reported a DNA-independent interaction between Yap1 and Skn7 in *S. cerevisiae* (92), and in *C. glabrata*, Yap1 and Skn7 cooperatively bind to the upstream region of core oxidative stress genes (90). In *S. pombe*, a role for two-component mediated phosphorylation of the Skn7 homologue in responses to high levels of H_2O_2 stress has been uncovered (93). A recent review summarizes the functions of Skn7 and its roles in fungal virulence (94).

In addition to Yap1 and Skn7 are the bZip factors of the ATF/CREB family. The best characterized is the Atf1 transcription factor in the model yeast *S. pombe*. In response to oxidative stress, Atf1 is hyperphosphorylated by the Sty1 SAPK (95). This stabilizes this transcription factor, which is vital for its function in

oxidative stress-induced gene expression (96, 97). Analogous transcription factors shown to play roles in oxidative stress-mediated gene expression include Atf1 in *C. neoformans* (98), Moatf1 in *Magnaporthe oryzae* (99), and AtfA in *A. nidulans* and *A. fumigatus* (100, 101). Moreover, there is emerging evidence to support the general concept that such transcription factors are targets of fungal SAPK pathways (101, 102).

Although the Hog1 SAPK in *S. cerevisiae* is dispensable for oxidative stress responses (20, 103), homologues play important roles in oxidative stress tolerance in many other fungi. These include the model yeast *S. pombe* (104); the filamentous fungus *A. nidulans* (101); a number of human pathogenic fungi including *C. albicans* (105), *C. neoformans* (58), and *A. fumigatus* (106); and the plant pathogens *Bipolaris oryzae* (107) and *Fusarium graminearum* (108). The Sty1 pathway in the model yeast *S. pombe* has provided key insight into the oxidative stress-mediated activation of such pathways. Sty1 is robustly phosphorylated in response to oxidative stress and plays a major role in the regulation of the oxidative-stress-induced transcriptome (91). In *S. pombe*, a two-component signaling system operates to relay H_2O_2, and not osmotic, stress signals to the Sty1 SAPK module. The Mak2 and Mak3 histidine kinases contain redox-sensing PAS and GAF domains, which are essential for the relay of H_2O_2 signals to Sty1 (93). In addition, protein oxidation also regulates the H_2O_2-induced activation of Sty1.

The redox-sensitive peroxiredoxin enzyme Tpx1 is essential for H_2O_2-mediated Sty1 activation and, because intermolecular disulfide bonds are formed between conserved cysteine residues in Sty1 and Tpx1 following H_2O_2 stress, it appears that Tpx1 may regulate Sty1 function directly (109). Mechanisms underlying H_2O_2-mediated SAPK activation have also been explored in *C. albicans*. *C. albicans* Hog1 is robustly activated following exposure to H_2O_2 (70), and *hog1*Δ cells are sensitive to a range of ROS (105, 110), despite a limited transcription role (70). Similar to that in *S. pombe*, oxidative stress-induced Hog1 phosphorylation is drastically reduced in cells lacking the Ssk1 response regulator (55), although no similar roles have been found for the upstream histidine kinases (53, 111). Both the redox-sensitive peroxiredoxin Tsa1 and thioredoxin Trx1 enzymes are vital for H_2O_2-induced Hog1 activation in *C. albicans* (75). Thus, protein oxidation appears to be an important mediator of both *C. albicans* and *S. pombe* SAPK activation following oxidative stress. Mechanistic details regarding oxidative-stress-mediated SAPK activation in other fungi are lacking, although in *A. nidulans* it has been shown that the SskA response regulator is essential for oxidative stress-induced activation of the SakA SAPK (112).

Nitrosative Stress

Fungal cells experience nitrosative stress when they are exposed to relatively high levels of reactive nitrogen species (RNS). These RNS include nitric oxide (·NO) and its derivatives peroxynitrite ($ONOO^-$, which is formed by the reaction of nitric oxide with superoxide, $O_2^{\cdot -}$), nitrite (NO_2^-), nitrogen dioxide ($\cdot NO_2^-$), and nitrate (NO_3^-). Fungi are confronted by RNS in the soil, as well as in mammalian hosts, where these reactive molecules are components of the phagocytic armory used to combat microbial infection (113–115). Therefore, the protective responses of fungal pathogens against nitrosative stress are important for pathogenicity and have been examined in some detail, for example, in *C. albicans* and *C. neoformans* (116, 117). In contrast, for plant pathogens such as *Blumeria graminis*, *Botrytis cinerea*, and *M. oryzae*, NO has been shown to promote the spread of infection in plant hosts. These effects might be mediated by the action of NO as a developmental signal (118–120) but could also be due to the influence of fungus-derived NO on the behavior of the plant host (121).

Excess RNS damage proteins by reacting with thiols, metal centers, and tyrosine residues. Fungi protect themselves by buffering RNS with antioxidants such as glutathione and by inducing mechanisms to detoxify the RNS and repair the damage they cause. NO reacts with glutathione (GSH) to generate S-nitrosoglutathione (GSNO). To restore redox homeostasis, fungi recycle this GSNO to GSH via glutathione disulfide. This is achieved through the evolutionarily conserved enzymes GSNO reductase (GSNOR: Fdh3) and glutathione reductase (122–124). NO detoxification to nitrate is mediated by nitric oxide oxidoreductases (e.g., *S. cerevisiae* Yhb1), which are conserved enzymes that are members of the flavohemoglobin family (125).

While the processes that protect against RNS are evolutionarily conserved, the regulatory mechanisms that induce these processes in response to RNS appear to have diverged. For example, while RNS exposure triggers global changes in the expression profiles of *S. cerevisiae* and *C. albicans* that include the induction of Yhb1 and glutathione synthesis (116, 126), seemingly unrelated transcription factors drive these changes. In *S. cerevisiae*, *YHB1* expression is induced by the transcription factor Fzf1 (126) (Fig. 1), whereas Cta4 activates *YHB1* in *C. albicans* (127). Nevertheless, the outcomes are similar: these transcription factors both

promote Yhb1 and glutathione expression and, hence, nitrosative stress resistance in these fungi. For the fungal pathogen *C. albicans* the deletion of *YHB1* or its activator *CTA4* attenuates virulence slightly (116, 127, 128). Similarly, inactivation of the nitric oxide oxidoreductase (Fhb1) or the GSNOR (Gno1) in *C. neoformans* attenuates the virulence of this pathogen (129). NO production by the host does not seem to be a major factor in limiting fungal virulence (116). Nevertheless, the available data suggest that RNS detoxification does contribute to fungal virulence.

Cell Wall Stress

The fungal cell wall is a dynamic structure that is continually remodeled during cell growth and division. The cell wall represents ~25% of the yeast cell dry weight, underscoring the extensive metabolic commitment to support this elaborate structure, which provides the key interface for mediating interactions with the environment (130). Fungal cell wall architecture involves layers of polysaccharides and glycoproteins, although the specific composition varies across species. Typically, a matrix of chitin, β-1,3-glucan, and β-1,6-glucan constitutes the core inner layer, with mannans and other mixed glycans and glycoproteins prevalent in the outer layer. Cell walls provide crucial protection against changes in external osmotic potential and can confer resistance to infection and to degradation by soil predators such as amoebae and protists. These adaptive advantages may contribute to the emergence and maintenance of cell walls in the fungal kingdom (131). Cell walls serve not only as a protective shell, but also as a means to modulate immune recognition. Fungal cell wall glycans, glycolipids, and proteins that are absent from mammals activate a wealth of immune recognition mechanisms, and the dynamic exposure of such pathogen-associated molecular patterns can modulate immune recognition (132). As a consequence, perturbation of fungal cell wall architecture can potentiate immune responses and induce lethal cell wall stress. Molecules that potently inhibit fungal cell wall biosynthesis have been elaborated in nature as with the echinocandins and exploited in modern medicine, with semisynthetic derivatives now a front-line treatment for fungal infections (133).

Fungi have evolved complex cellular circuitry to sense and respond to cell wall stress. Although details vary among fungi, the core architecture is broadly conserved. Cell wall stress is typically sensed at the plasma membrane via cell surface sensors, which include Wsc1, Wsc2, Wsc3, Mid2, and Mtl1 in *S. cerevisiae* (130). This stimulates nucleotide exchange on the small G-protein Rho1, which orchestrates cell wall integrity signaling. Downstream effectors of Rho1 coordinate synthesis of β-glucans, transcriptional control of cell wall genes, polarization of the actin cytoskeleton, and targeting of secretory vesicles. The most well-established pathway through which cell wall integrity signals are transduced from Rho1 is the MAPK cascade that includes Pkc1, Bck1, Mkk1/2, and Mpk1/Slt2 in *S. cerevisiae* (17). Although this cascade provides a powerful mechanism to amplify cell surface signals and coordinate highly sensitive responses, additional robustness is integral to ensure maintenance of cell wall physiology and is achieved by complex genetic interaction networks. These networks can enable compensatory responses to cell wall perturbations. For example, activation of chitin synthesis suppresses the antifungal activity of echinocandins (134–136), which inhibit biosynthesis of β-1,3-glucan. The highly connected genetic networks that control cell wall stress response circuitry can be activated by diverse environmental stresses, providing a powerful strategy to rapidly mobilize protective mechanisms.

Environmentally contingent hubs of cellular signaling are crucial for orchestrating cell wall stress responses. An excellent example is the molecular chaperone Hsp90. Hsp90 modulates the stability and function of diverse regulators of cellular signaling, thereby enabling responses to a myriad of stresses, including perturbation of the cell wall (29, 137, 138) (Fig. 1). Hsp90 enables responses to cell wall stress at least in part by modulating cell wall integrity signaling. Hsp90 stabilizes the terminal MAPK in the cell wall integrity pathway, Slt2 in *S. cerevisiae*, and Mkc1 in *C. albicans* (139–141). Hsp90 also stabilizes an additional MAPK that is implicated in cell wall remodeling: Cek1 (29). Compromise of Hsp90 function leads to depletion of these kinases and hypersensitivity to cell wall stress. Additional Hsp90 client proteins important for cell wall remodeling are the protein phosphatase calcineurin and kinase Hog1. Signaling through the cell wall integrity pathway, Hog1, and calcineurin coordinately regulates the synthesis of chitin in response to stress induced by perturbation of the cell wall or cell membrane (142). Chitin levels are dramatically increased in response to echinocandins, which provides protection that enables cells to cope with cell wall damage (134). Cell damage induced by various genetic or environmental insults can also be buffered by activation of other core cellular signaling pathways such as the cyclic AMP protein kinase A cascade (17). Although the molecular details have been explored in the greatest depths in *S. cerevisiae*, many conserved principles of coordinate control

of cell wall stress response are emerging from studies in diverse fungi (143–146).

There is broad therapeutic potential of targeting core regulators of cell wall stress responses as a strategy to enhance the efficacy of antifungal drugs that target the cell wall, as with the echinocandins. This potential is illustrated by the discovery that inhibition of Hsp90 of calcineurin enhances the efficacy of echinocandins against diverse fungal pathogens in multiple models of infection (137, 144, 147). The therapeutic challenge of exploiting these conserved eukaryotic cellular regulators as targets for antifungal drug development lies in the development of molecules that can distinguish the pathogen from the host. Achieving this goal can be facilitated by structure-guided drug design (148). As a complementary approach to targeting regulators of cell wall stress response, systematic screens for molecules that potentiate the activity of echinocandins provide a powerful strategy to enhance the efficacy of our limited arsenal of antifungal drugs and thwart the emergence of drug resistance (149).

pH Stress

Alkaline pH imposes several stresses on fungi. One of the most significant relates to nutrient acquisition. At high extracellular pH, the establishment of electrochemical gradients across the plasma membrane for nutrient transport and ATP synthesis is more difficult (150). Moreover, the solubility and biological availability of essential elements such as iron are dramatically reduced at high pH. The finding that the addition of micromolar concentrations of copper or iron ions significantly improves the growth of *S. cerevisiae* at high pH suggests that these two elements are limiting factors for growth under alkaline pH conditions (151).

An important aspect of pH regulation is the ability to regulate gene expression in response to ambient pH, which allows fungi to synthesize the environmentally appropriate gene products, particularly secreted proteins. This ability has practical implications, for example, in the production of secondary metabolites or for fungal pathogenicity on plant and animal hosts (152, 153). Environmental pH also has profound effects on fungal development. For example, in *C. albicans* a shift from acidic to neutral-alkaline pH promotes the transition from yeast to filamentous growth (152).

The best-known alkaline pH-responsive signal transduction mechanism in fungi is the Pal/Rim pathway (Fig. 1). This pathway has been extensively studied in *A. nidulans* (Pal) and *S. cerevisiae* (Rim). In *A. nidulans*, the Pal pathway sequentially involves the proteins PalH, PalI, PalF, PalC, PalA, and PalB. Moreover, the pathway encompasses a number of co-opted components of the multivesicular body and endosomal sorting complexes required for transport (153). Activation of the pathway by alkaline pH leads to proteolytic activation of the zinc finger transcription factor PacC/Rim101 (154, 155). PacC undergoes two successive C-terminal proteolytic cleavages from the full-length 72-kDa to the processed 27-kDa active form, the first of which is pH-dependent and carried out by the signaling protease PalB (153).

PalH/Rim21, a seven-transmembrane-domain plasma membrane protein, is thought to function as a pH receptor. PalH forms a complex with the arrestin-like protein PalF/Rim8, which is ubiquitinated in an alkaline pH-dependent manner and recruits endosomal sorting complexes required for transport-I Vps23, thereby creating multiple docking sites for the downstream signaling components (156–159).

An unanswered question is how PalH/Rim21 senses alkaline ambient pH. The membrane potential of the yeast plasma membrane is mainly generated by differences in proton concentration between the inside and outside of the cell. External alkalization therefore leads to impaired phospholipid flipping and plasma membrane depolarization by collapsing the proton electrochemical gradient. Interestingly, it was shown that the Rim101 pathway in yeast can be activated in a pH-independent manner by either protonophore treatment or depletion of phosphatidylserine in the inner leaflet of the plasma membrane, both of which cause plasma membrane depolarization similar to external alkalization. This activation is dependent on Rim21, suggesting that plasma membrane depolarization is a key signal sensed by Rim21 (159). Moreover, a recent study suggests that alterations in lipid asymmetry cause changes in lipid composition and local charge on the inner leaflet, leading to dissociation of the Rim21 complex from the plasma membrane and recruitment of downstream proteins (160). Thus, Rim21 senses external alkalization, as well as altered lipid asymmetry. It was proposed that Rim21 uses its flexible C-terminal cytosolic tail like an antenna to monitor the status of membrane lipid asymmetry (160).

To achieve infection, pathogenic fungi must adapt to wide variations in the ambient pH of host tissues, which in humans, can vary from 2 to >8 depending on the niche. Early studies of *C. albicans* revealed that genes encoding two functionally redundant cell wall β-glycosidases, *PHR1* and *PHR2*, display divergent pH-dependent expression patterns and virulence functions in cell wall remodeling. While *PHR1* is expressed preferentially at neutral-alkaline pH and is required for

systemic infection, *PHR2* is expressed preferentially at acidic pH and is required for vaginal infection (161). These pH-dependent expression patterns are dependent on PacC/Rim signaling (162). More recently, multiple roles for the PacC/Rim pathway during human colonization and infection have been established in *C. albicans*, including filamentation, adhesion to host cells, tissue invasion, iron acquisition, and protease secretion (152). The role of PacC/Rim101 in mammalian infection is conserved in filamentous ascomycetes such as *Fusarium oxysporum* and *A. fumigatus* (163, 164). In the basidiomycete human pathogen *C. neoformans* the RIM pathway is also involved in pathogenicity (165), although the function of PalH/Rim21 appears to be carried out by a distinct membrane protein (166).

Alkaline pH signaling is also relevant in other fungus-host interactions. For example, deletion of PacC in the nematophagous fungus *Clonostachys rosea* and the insect pathogen *Metarhizium robertsii* results in attenuated virulence (167, 168). In plant pathogens, the role of PacC in virulence varies depending on the pathogen-host system. While it contributes to virulence in the rice blast fungus *M. oryzae* and the fruit pathogen *Penicillium expansum*, it appears to function as a negative virulence regulator in the vascular wilt fungus *F. oxysporum* (169–171).

Besides the Pal/Rim pathway, additional cell signaling pathways function in fungal adaptation to neutral/alkaline pH. The calcium-dependent protein phosphatase calcineurin and its downstream transcription factor Crz1 are required for growth at alkaline pH and for fungal virulence on animal and plant hosts (172–176). Crz1 also mediates tolerance to high cation concentrations (177). Mds3, a negative regulator of the TOR pathway, promotes adaptation to neutral/alkaline pH as well as virulence-related morphogenesis in *C. albicans* (178). Moreover, mutations in the cell wall integrity MAPK cascade confer sensitivity to alkaline stress, and alkalinization results in rapid and transient phosphorylation of the Slt2 MAPK in *S. cerevisiae* (179). This suggests that alkaline pH stress profoundly affects the composition of the fungal cell wall.

Recently, a novel signaling pathway was identified which is required for resistance to alkaline pH and cation stress in *A. nidulans* (176). This pathway, which appears to be specific to filamentous fungi, is defined by the transcription factor SltA and the serine protease SltB. Activation of SltA requires proteolytic cleavage and removal of the N-terminal domain by SltB and phosphorylation of the functional C-terminal moiety (180). Interestingly, while the SltA pathway is conserved in filamentous fungal pathogens, its role in infection remains to be determined.

Weak Acid Stress Response

The weak acid stress response has been largely studied in the context of food spoilage, but it is likely to be highly relevant to environmental and pathogenic fungi that occupy acidic niches or that are exposed to phagocytic attack. Weak organic acids such as acetic, propionic, sorbic, and benzoic acids impose stress on fungal cells when the environmental pH lies below the pK_a of the acid in question (i.e., below pH 4.8 for acetic acid). Below its pK_a, a weak acid is in its associated nonpolar form and is better able to cross the plasma membrane. Acetic acid enters the *S. cerevisiae* cell via the aquaglyceroporin Fps1, whereas propionic, sorbic, and benzoic acids appear to diffuse passively across the plasma membrane (181). Once they enter the higher pH of the cytoplasm, they dissociate into the free acid anion and proton (H^+). The equilibrium of this reaction drives the accumulation of the acid anion and protons and, consequently, acidification of the cytoplasm. Weak acid stress is imposed partly by this cytoplasmic acidification and partly by the accumulation of the organic anion, which can impose toxic effects on yeasts and molds (182, 183). For example, sorbic acid appears to exert membrane-active antimicrobial effects in *S. cerevisiae* (182).

Fungal cells respond to weak acid stress by attempting to maintain their intracellular pH and by exporting the organic anion. In *S. cerevisiae* these tasks are executed by Pma1 and Pdr12, respectively (184, 185). Pma1 is the plasma membrane H^+-ATPase proton pump. This pump is essential for growth and for the restoration of cytoplasmic pH, which is an energy-demanding process (186). Pdr12 is an ATP-binding cassette transporter that mediates efflux of the organic anion from the yeast cell. Pdr12 is also important for the restoration of cytoplasmic pH during sorbic and benzoic acid stress (187).

In *S. cerevisiae*, resistance to acetic acid stress is dependent on Hog1 signaling. Hog1 downregulates the Fps1 aquaglyceroporin, thereby reducing acetate accumulation (181), and activates the Haa1 transcriptional regulon, which includes genes encoding membrane stress proteins (188). Meanwhile, resistance to propionic, sorbic, and benzoic acids depends on the induction of *PDR12* (184) via the transcription factor War1 (189, 190). War1 is potentially regulated by direct binding of the organic anion, thereby precluding a requirement for upstream signaling.

Analogous regulatory mechanisms appear to exist in some yeasts. For example, Hog1 mediates sorbic acid

resistance in *C. glabrata* (191), and resistance to this weak acid in *C. albicans* is dependent on a War1 orthologue (192) as well as the Msn2/4-like transcription factor Mnl1 (193). However, alternative mechanisms seem to mediate weak acid stress resistance in other yeasts, most notably the food spoilage organism *Zygosaccharomyces bailii*, which displays high levels of weak acid stress resistance. In this species, survival in the face of weak acid stress appears to be dependent on population heterogeneity: a proportion of cells that display low cytoplasmic pH, and therefore reduced weak acid accumulation, give rise to a population of resistant cells (194). Rather than inducing a Pdr12-like weak acid exporter, *Z. bailii* degrades sorbate and benzoate, exploiting them as a carbon source (184).

Core Stress Response

In the preceding sections we discussed fungal responses to individual stresses. Here we describe how exposure to different types of stress can lead to similar responses via what has been termed the core stress response (CSR). Genome-wide transcript profiling studies first revealed the existence of CSRs in the model yeasts *S. cerevisiae* (20, 67) and *S. pombe* (68), in the pathogenic fungus *C. glabrata* (71), and to a lesser extent in *C. albicans* (69, 70). Formally, the CSR describes a set of genes that are commonly regulated in response to diverse types of stress (Fig. 2).

In *S. cerevisiae*, between 200 and 300 genes were found to be upregulated in response to diverse stresses including heat shock, osmotic stress, oxidative stresses, increases or decreases in pH, and amino acid starvation (20, 67). In addition, between 300 and 600 genes were commonly downregulated following exposure to these diverse stress treatments (20, 67). Thus, in *S. cerevisiae*, the CSR involves approximately 10 to 14% of the yeast genome. Similar numbers of genes were reported to be regulated in the closely related, but pathogenic, yeast *C. glabrata* following exposure to glucose starvation and osmotic, oxidative, and heat stresses (71). Furthermore, in the divergent yeast *S. pombe*, approximately 140 genes are commonly upregulated and 100 downregulated in response to a range of stresses including osmotic, oxidative, heavy metal, DNA damage, and heat stress (68). Common processes were represented in the induced core stress gene sets such as carbohydrate metabolism, protein folding and degradation, redox regulation, and DNA repair. In contrast, repressed genes were associated with energy-consuming and growth-related processes, including RNA processing, transcription and translation, and biosynthesis of ribosomes and nucleotides. Interestingly, the pathogenic fungus *C. albicans* mounts a significantly smaller CSR than those described above, because only 24 and 37 genes were commonly induced or repressed, respectively, by osmotic, oxidative, and heavy metal stress (70). Despite this, the CSR genes in *C. albicans* do belong to some of the same functional categories as those in *S. cerevisiae*, *S. pombe*, and *C. glabrata*, suggesting that some of the processes involved in the CSR are evolutionarily conserved.

In *S. cerevisiae*, different signaling pathways and transcription factors converge to control a common set of stress genes, although the functionally redundant Msn2 and Msn4 $(C_2H_2)_2$ zinc finger transcription factors play a major role (20, 67). Consistent with this, many of the CSR genes carry a stress response element within their promoters, to which Msn2 and Msn4 bind (20, 67). This Msn2/4-mediated CSR forms the basis of the previously characterized general stress response in *S. cerevisiae*, which is composed of an Msn2/4-stress response element regulon that is activated in response to diverse stresses (195). Msn2/4 rapidly accumulate in the nucleus following a range of nutrient and stress conditions (196, 197) and are subject to complex regulation by several pathways (198). However, a number of other factors also regulate the CSR in *S. cerevisiae*. In response to osmotic stress, the Hog1 SAPK contributes to the regulation of CSR genes (20), likely through the Hot1 transcription factor (59), whereas the Yap1 AP-1-like transcription factor contributes to CSR gene induction postoxidative stress (20). In addition, the Mec1 DNA-damage specific pathway contributes to CSR gene regulation in response to DNA damaging agents (199).

Recently, it has been shown that the Msn2 transcription factor also makes a major contribution to the regulation of CSR genes in *C. glabrata* (71). Consistent with this, in this pathogenic yeast, Msn2 rapidly localizes to the nucleus following glucose starvation and in response to osmotic, oxidative, or heat stress (71). In stark contrast, homologues of Msn2 and Msn4 do not have the same broad stress-protective roles in *C. albicans* (193, 200), which may contribute to the relatively small CSR seen in this fungus (70). Moreover, in *S. pombe*, which lacks close homologues of Msn2/4, the CSR is regulated by a different mechanism. In this yeast, the Sty1 SAPK is activated in response to diverse stress conditions including osmotic, oxidative, and heat stress; nutrient limitation; UV light; and cold stress (201). Following activation, Sty1 accumulates in the nucleus, where it phosphorylates the Atf1 transcription factor, leading to its activation and stabilization (95–97). Sty1 and, to a lesser extent, Atf1 are the major

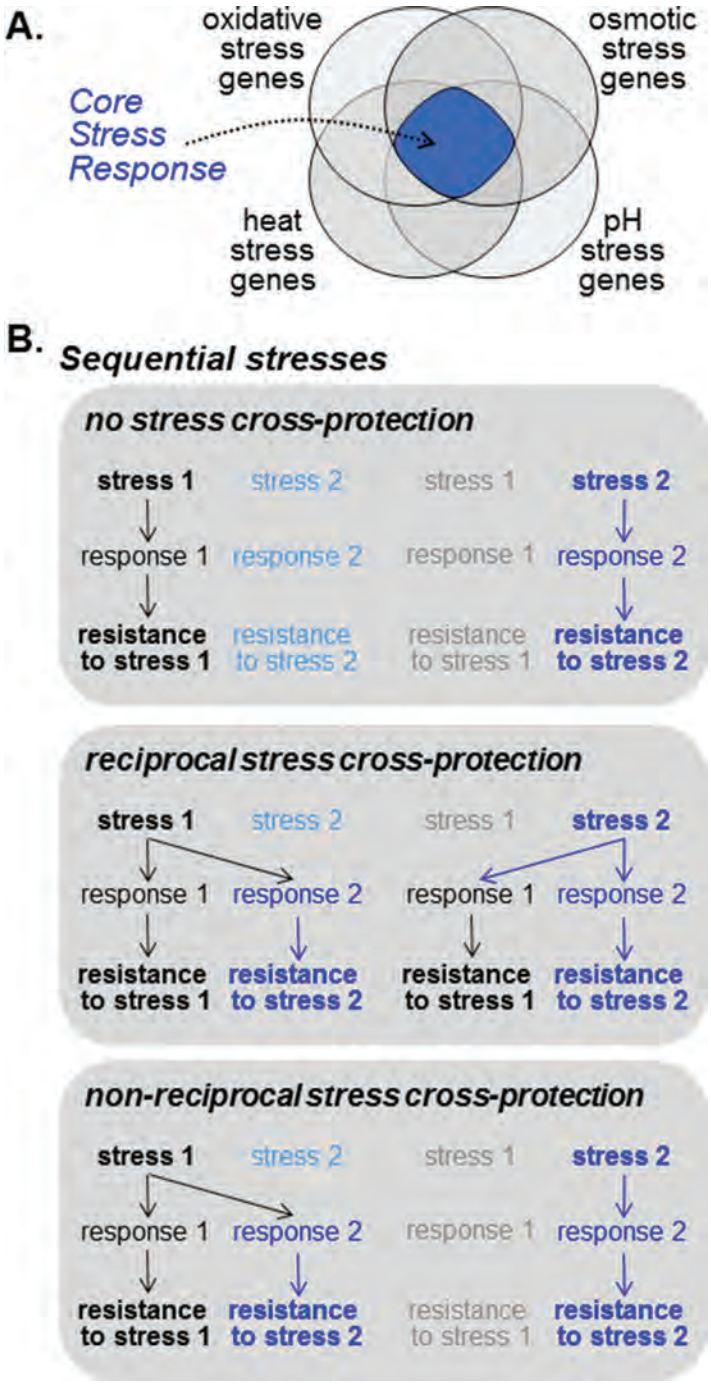


Figure 2 The CSR can lead to stress cross-protection. (**A**) CSRs, which have been defined by genome-wide transcriptional profiling, represent the set of genes that is commonly up- or downregulated by different types of stress (see text). This Venn diagram illustrates the conceptual overlap between these sets of genes, highlighting the core stress genes. (**B**) A CSR can lead to stress cross-protection during exposure to sequential stresses; i.e., cells that are exposed to one type of stress can then display elevated resistance to a subsequent stress of a different type (see text). In some cases no cross-protection is observed. In other cases it is observed, but this cross-protection can be reciprocal or nonreciprocal. This can depend on the nature and dose of the initial and subsequent stress.

regulators of the CSR in *S. pombe* (68). It is interesting that CSR genes implicated in stress defense are dependent on Sty1 and Atf1, whereas CSR genes with regulatory functions are induced by Sty1 independently of Atf1 (68).

Similar to the Sty1 SAPK in *S. pombe*, the Hog1 SAPK in the fungal pathogen *C. albicans* is activated in response to diverse stresses (110). Significantly, a CSR was only observed after treating cells with three stress conditions—osmotic, oxidative, and heavy metal stress—that activate Hog1 (69, 70). Intriguingly, however, Hog1 regulated the transcriptional responses to osmotic and heavy metal stresses, but not to oxidative stress, and this was reflected in the role of Hog1 in the regulation of *C. albicans* CSR genes. Instead, the Cap1 AP-1-like transcription factor regulated the *C. albicans* CSR following oxidative stress (70). Thus, the *C. albicans* SAPK pathway functions in parallel with other pathways to regulate the core transcriptional response to stress. Hence, although aspects of a CSR are conserved across the fungal kingdom, the mechanisms underlying its regulation have diverged significantly.

What is the physiological role of a CSR? Earlier studies in *S. cerevisiae* revealed a phenomenon called "stress cross-protection," in which exposure to a nonlethal dose of one stress provided significant protection from the subsequent exposure of a potentially lethal dose of a second unrelated stress (202) (Fig. 2). Such stress cross-protection was impaired in the presence of cycloheximide, illustrating a role for new protein synthesis (202). The Msn2/4-mediated general stress response in *S. cerevisiae*, together with the identification of the CSR, probably accounts for the observed stress cross-protection in several fungi (68, 71, 110). Interestingly, subsequent studies have revealed that the CSR triggered by the initial low stress dose is not required to survive this stress but instead provides protection against the second stress (203). Furthermore, the actual genes and processes necessary to acquire resistance to the same severe stress are different depending on the nature of the initial mild stressor. In other words, the mechanism of stress cross-protection is determined by the initial stress (204). This probably underlies previous findings that stress cross-protection is context dependent and not always reciprocal (203) (Fig. 2).

But why might stress cross-protection have evolved? Some microbes occupy reasonably predictable niches in which one environmental input is generally followed by a second input. In such cases, fungi that have evolved to anticipate the second input following exposure to the first would have a fitness advantage (205). Domesticated brewing yeasts provide an excellent example of this "adaptive prediction" because, as they ferment sugars, they become exposed to increasing ethanol concentrations (input 1) and then, when the sugars are exhausted, they switch to respiratory metabolism and become exposed to oxidative stress (input 2). Presumably, as a consequence of this environmental predictability, *S. cerevisiae* has evolved to activate oxidative stress genes following exposure to ethanol (206). Asymmetric adaptive prediction appears to have evolved in other fungi such as *C. albicans* (207, 208). Unlike *S. cerevisiae*, this pathogen displays increased resistance to acute oxidative stress following exposure to glucose (209), which possibly reflects anticipation of phagocytic attack after entry to the bloodstream. Therefore, it is conceivable that CSRs have evolved as a result of adaptive prediction.

ADAPTING TO STRESS IN NATURAL ENVIRONMENTS

Combinatorial Stress Responses

Our understanding of stress responses in yeast has been shaped largely by the study of individual stresses or, as discussed in the previous section, how the prior exposure to one stress can provide stress cross-protection against a second unrelated stress. However, the diverse environments that fungi occupy are complex and dynamic, and it is conceivable that fungi will often be exposed to multiple stresses. At times these stresses may be imposed sequentially, in which case stress cross-protection may facilitate survival. However, at other times, a fungus may be exposed simultaneously to multiple stresses, termed "combinatorial stress." Recent studies have revealed that certain combinations of stresses are particularly potent in terms of their ability to kill functionally divergent model (*S. cerevisiae*, *S. pombe*) and pathogenic (*C. albicans*, *C. glabrata*) yeasts (210, 211). Notably, all of these species are acutely sensitive to combinations of cationic and oxidative stresses (210). Pathogenic fungi encounter this combination of stresses following phagocytosis; microbes are exposed to high levels of ROS generated by the respiratory burst (212), and the resulting accumulation of anionic charge is compensated for by a rush of potassium (K^+) ions into the endocytic vacuole, which simultaneously imposes a cationic stress (212).

Strikingly, studies investigating the mechanistic basis underlying the exquisite sensitivity of *C. albicans* to such combinations of stress have revealed that exposure to cationic stress prevents this pathogen from mounting an oxidative stress response. The oxidative

stress regulon in *C. albicans* is largely regulated by the Cap1 AP-1-like transcription factor (213), and Cap1 fails to be activated following exposure to combinatorial oxidative and cationic stress (210, 214). This phenomenon, which has been termed "stress pathway interference" (210) (Fig. 3), contrasts starkly with that of stress cross-protection in which exposure to one stress protects against the subsequent exposure to a different stress (103). Exposure of *C. albicans* to H_2O_2 triggers the rapid oxidation of Cap1, and this masks the nuclear export sequence, resulting in the rapid nuclear accumulation of Cap1 and the induction of Cap1-dependent genes. However, cationic stress inhibits this Cap1-mediated oxidative stress response in two ways. First, cations inhibit catalase activity, which triggers significant increases in intracellular ROS levels following combinations of cationic and oxidative stresses (210). Such high levels of ROS trap Cap1 in a partially oxidized form that fails to induce target antioxidant-encoding genes (214). Second, cations stimulate the interaction of Cap1 with the nuclear export factor Crm1, which results in significant delays in the H_2O_2-induced nuclear accumulation of this transcription factor. Importantly, the cationic stress-mediated inhibition of oxidative stress responses contributes to the fungicidal potency of human neutrophils, because effective killing of *C. albicans* is dependent on the combinatorial effects of the oxidative burst and cationic fluxes (210). These findings may also explain the lack of expression of *C. albicans* oxidative stress genes in certain host niches, such as during systemic infections of the kidney, despite the presence of neutrophil infiltrates (215).

Although to date, studies have focused on combinatorial oxidative plus cationic stress, it is feasible that other stress combinations will influence stress outputs (207). Indeed, we have found that pH has drastic effects on the oxidative stress tolerance of a number of fungi (J.Q., A.J.P.B., unpublished). This is an exciting new area in the field of stress responses that is likely to be of broad relevance across the fungal kingdom due to the complexity of natural environments.

Dynamics of Stress Responses

Our perspectives of fungal stress responses and stress resistance have been shaped largely by our experimental approaches. For example, plate assays, which are widely used to examine stress resistance, often do not differentiate between the ability of a strain to survive immediately following exposure to an acute stress and its ability to adapt and resume growth in the longer term. Also, the availability of powerful molecular genetics and genomics approaches has led to major advances in our understanding of fungal stress responses at the gene and protein levels, but the metabolic changes that underlie stress adaptation have received less attention. Yet these changes play vital roles in fungal stress resistance.

The resistance of yeast cells to stress is enhanced by increases in metabolic flux toward the generation of antioxidants such as glutathione and stress protectants

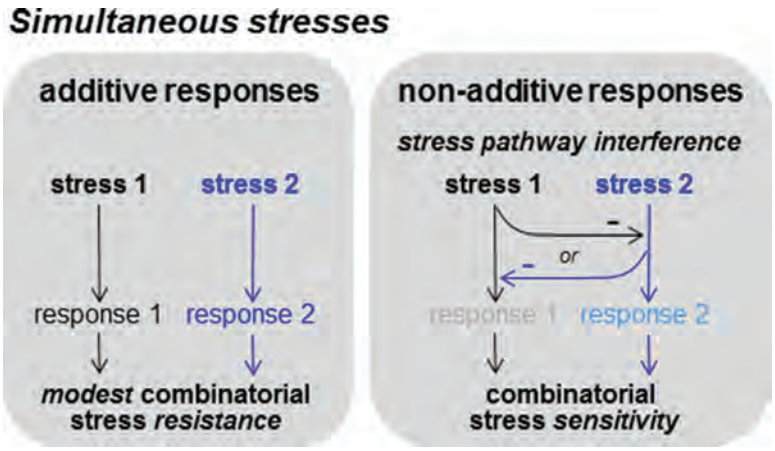


Figure 3 Exposure to combinatorial stresses can yield nonadditive outputs. Simultaneous exposure to some combinations of stress (i.e., certain combinatorial stresses) can yield additive outputs if there are no significant interactions between the stress pathways that mediate these responses. However, for some combinatorial stresses (see text), stress pathway interference can block the normal response to one of the imposed stresses, leading to combinatorial stress sensitivity. We are unaware of any examples of the opposite effect, where stress pathway enhancement might lead to elevated levels of combinatorial stress resistance.

such as trehalose (122, 216–219). Like the accumulation of glycerol in response to hyperosmotic stress (36), these increases in glutathione and trehalose levels are mediated in large part by changes in gene expression and enzyme synthesis and hence are slow. Other metabolic responses are much faster. For example, there is a rapid shift in metabolic flux from lower glycolysis toward the pentose phosphate pathway upon exposure to oxidative stress (72). This metabolic shift, which is mediated through the sensitivity of glyceraldehyde-3-phosphate dehydrogenase to oxidative inactivation (220), increases the NADPH synthesis and hence the availability of protective reducing equivalents (72). This metabolic response to oxidative stress occurs within seconds, preceding transcriptional responses to oxidative stress (221).

Clearly, different aspects of a fungal adaptive response take place over different timescales (36) (Fig. 4). Initial metabolic and biophysical responses can occur within seconds to minutes (37, 221). Signal transduction is activated rapidly, within minutes, and often remains active for tens of minutes (36). This triggers changes in gene expression: transcript levels often rise within 5 minutes and, depending on the stability of the mRNA, can remain elevated for tens of minutes. Resultant changes in enzyme levels are often observed in tens of minutes and can last for hours, depending on the stability of these proteins. Consequently, the accumulation metabolites such as glycerol can take tens of minutes to an hour.

While these general principles hold, the dynamics of a stress response are strongly influenced by the dose. Large single doses are often applied to experimentally dissect a stress response *in vitro*. However, in reality, that stress response might have evolved to maintain

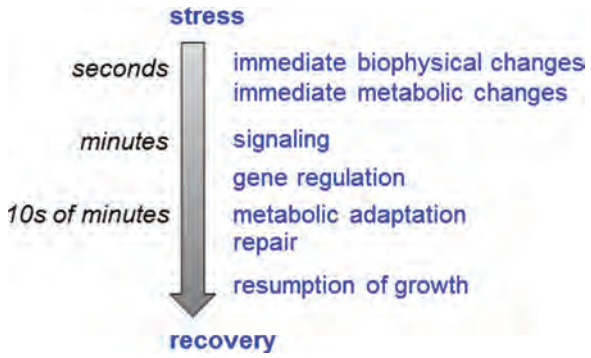


Figure 4 Different aspects of stress adaptation occur over different timescales. This generic figure summarizes this principle of an environmental insult such as osmotic stress (see text). However, some stresses may include adaptation mechanisms that occur over other timescales.

cellular homeostasis in the face of less acute but multiple challenges. For example, researchers often use large acute heat shocks to study thermal adaptation, although many fungi encounter less dramatic thermal fluctuations in the wild. Also, researchers generally examine the impact of a single dose, and yet fungi can face repetitive doses of certain stresses in some habitats (e.g., repetitive hypo-osmotic shock during rainfall). The mathematical modeling of stress responses permits the analysis of the vast theoretical space represented by variations in the dose, exposure time, and frequency of a stress. Indeed, mathematical modeling is already improving our appreciation of the dynamics of stress responses, the influence of stress dose, and their impact on stress adaptation in fungi (36, 62, 222, 223). These dynamics are significant because they influence the length of time for which the molecular memory of a stress is retained and hence the period over which stress adaptation provides protection against a subsequent stress (224) (see "Core Stress Response," above).

Impact of Growth Conditions on Stress Resistance

Historically, the dissection of fungal stress responses has largely been performed under a relatively small set of experimental conditions to facilitate comparison between studies. This has influenced our perceptions of stress adaptation. For example, it is well known that changes in carbon source exert dramatic effects on stress resistance in *S. cerevisiae*. Yet stress adaptation in *C. albicans* has largely been examined using glucose-rich media, although this fungus generally inhabits glucose-poor niches. Temperature also affects stress responses (29).

A shift from glucose to a nonfermentative carbon source increases stress resistance in *S. cerevisiae*, partly through activation of the CSR. Glucose inhibits the CSR through Msn2/4 phosphorylation, which is mediated by Ras-cAMP-protein kinase A signaling (225). Phosphorylation of Msn2/4 prevents the nuclear accumulation of these transcription factors, thereby blocking their activation of core stress genes (196). Glucose also regulates *YAP1* (the AP1-like transcription factor central to the transcriptional response to oxidative stress) and *ENA1* (a P-type ATPase Na^+ pump required for cationic stress resistance) (226, 227).

Changes in carbon source also affect stress resistance in *C. albicans*. Exposure to glucose increases oxidative stress resistance by upregulating oxidative stress genes in this yeast (209). In contrast, growth on glucose decreases the resistance of *C. albicans* to osmotic and cell wall stresses, in large part through carbon source-

dependent changes in the preadapted state of the cell wall (37, 228). Consequently, as different host niches contain different carbon sources, the nature of a niche must determine the ability of *C. albicans* to counteract stresses in that niche. Not surprisingly, the virulence of this pathogen is influenced by the carbon source (228).

CONCLUSIONS AND PERSPECTIVES

It is clear that major advances have been made in our understanding of fungal stress adaptation over the past decades. However, much remains to be learned with regard to how cells respond to stress in their natural environments. Several issues should be addressed.

First, many gaps remain in our understanding of stress signaling pathways and of stress responses themselves, even for individual stresses in model fungi. Just one example is our understanding of how nitrosative stress responses are regulated, which is rudimentary compared to oxidative and osmotic stress signaling even in *S. cerevisiae*. The situation is worse for model ascomycete and basidiomycete pathogens such as *C. albicans* and *C. neoformans*. Their lifestyles differ markedly from *S. cerevisiae* and *S. pombe*, and differential evolutionary pressures appear to have driven regulatory rewiring and niche-specific tuning of stress responses, yielding different stress sensitivities and different patterns of stress cross-protection.

Second, our understanding of the dynamics of stress adaptation needs to improve, incorporating the immediate and long-term contributions of metabolic responses and biophysical changes alongside those driven by transcriptional and posttranslational gene regulation. This needs to be considered alongside the issue of population heterogeneity. The molecular basis for the differential stress sensitivity of genetically identical cells experiencing the same environmental conditions needs to be better understood. To achieve this we require experimental approaches that provide dynamic views of cell-to-cell variation at high resolution.

Third, more consideration needs to be given to the nature of the stresses that are encountered by a fungus in its natural habitat and the nature of the microenvironment(s) in which it must respond to these stresses. How acute is the stress, how long is the exposure, and how frequently is it encountered? This is significant because some fungal stress responses may have evolved to maintain cellular homeostasis in the face of modest but repetitive challenges rather than single acute doses (see "Dynamics of Stress Responses," above). Is the stress imposed in combination with other environmental insults? This should be considered because certain combinations of stresses can yield unexpected stress responses (see "Combinatorial Stress Responses," above). What is the temperature of the niche, and what nutrients are available? These factors are important because they strongly influence the preadapted state of the fungus and hence its ability to counteract the stress (see "Impact of Growth Conditions on Stress Resistance," above).

If these issues are addressed, there will be a paradigm shift in our understanding of fungal stress responses and their relevance to survival in natural environments.

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Thigmo Responses: The Fungal Sense of Touch

22

Mariana Cruz Almeida and Alexandra C. Brand

INTRODUCTION

The growth and development phases of most fungi take place on a two-dimensional surface or within a three-dimensional matrix. The fungal sense of touch is therefore critical for fungi in the interpretation of their environment and is often involved in the switch to a new developmental state. Contact sensing, or thigmo-based responses, include thigmo differentiation, such as the development of invasion structures by plant pathogens; thigmonasty, where contact with a motile prey rapidly triggers its capture; and thigmotropism, where hyphal growth direction is guided by physical features in the environment. Like plants and some bacteria, fungi grow as walled cells. How do fungi, as walled organisms, not only sense contact, but also interpret the signal in a developmental context?

The fungal sense of touch was first observed in 1937, when Drechsler recognized the role of thigmonasty in the predaceous activity of the nematode-trapping fungus, *Arthobotrys oligospora* (1). This fungus forms a specialized hyphal stalk ending in a loop comprising three "capture cells" that form an aperture of approximately 20 μm (Fig. 1A). When a migrating nematode enters the loop, the capture cells sense contact with the prey and dramatically inflate to three times their normal size within a tenth of a second (Fig. 1B). The constriction immobilizes the nematode, which is subsequently penetrated and digested by hyphae that develop from the encircling capture cells (1, 2). Over a century later, the fungal sense of touch was found to be essential for the recognition, exploration, and invasion of host surfaces by plant pathogens using thigmotropism, or contact-induced directional growth (Fig. 1C and D). Studies using inert materials patterned to mimic host-leaf topography showed that highly defined three-dimensional surface features elicited pathogenic behaviors, including host-specific directional growth responses and the morphological development of specialized invasion structures (thigmo differentiation) (Fig. 1E) (3, 4). More recently, the contact-induced behavior of the human pathogen, *Candida albicans*, has been investigated. Although the characteristics of the external signals received by the fungus *in vivo* are not fully understood, the loss of normal hyphal touch responses correlated with attenuated tissue penetration, damage, and virulence (5–7). Fungal contact sensing has been primarily recognized and studied in the context of predation and pathogenesis, but it is involved in many other fungal behaviors including interactions with "self," such as mating and anastomosis (e.g., fusion between specialist cells or vegetative hyphae), and with "non-self," such as the symbiotic association of mycorrhizal fungi with plant roots. There are likely to be many other contact-dependent behaviors that are so far unappreciated because of the highly varied, and mainly unseen, lifestyles of the estimated 3 to 5 million fungal species (8).

The study of contact-dependent behavior by fungi presents several challenges. First, a link must be observed between a specific stimulus and a consistent response output. Fungal cells are microscopic, but their activity is often observed at the population level when they cause visible harm to valued macro-organisms such as food crops, livestock, and humans. Next, an experimental system must be devised that captures and defines the relevant stimulus so that fungal responses can be studied. Bespoke microscopy methods coupled with molecular tools, microfabrication, and laboratory-based host model systems have provided considerable insights as to the stimuli sensed by fungi that induce

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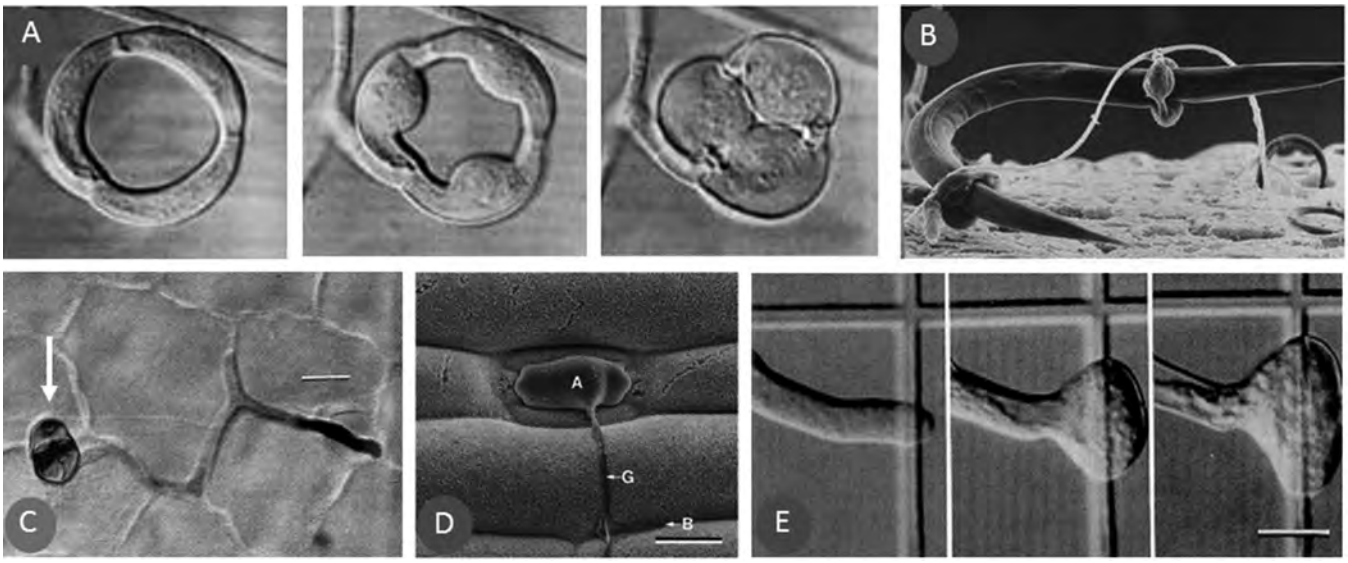


Figure 1 Thigmo responses play crucial roles in the fungal lifestyle. (**A**) Thigmonasty: On sensing exudate from nearby nematode prey, *Arthobotrys oligospora* hyphae produce a lateral peg and three consecutive capture cells that grow round until they contact the peg, with which they fuse to form a capture loop (1, 2) (reprinted from taxusbaccata.hubpages.com with permission of the author). (**B**) Contact with a nematode entering the aperture of the loop causes immediate swelling of the capture cells to immobilize the prey (reprinted from apsnet.org with permission of the author). Invasive hyphae then germinate from the contact zone of the capture cells to penetrate and digest the nematode. (**C**) Thigmotropism: Hyphae of the sooty blotch fungus, *C. trifolii*, follow interepidermal cell depressions on the surface of the host plant, *T. repens* (white clover), to locate a stoma, over which a penetrative appressorium is formed (arrow) (bar, 10 μm) (reprinted from *Mycological Research* [23] with permission of the publisher). (**D**) Germ tubes of the cereal rust fungus, *Puccinia hordei*, grow perpendicularly across depressions in the surface of its host barley leaf to locate stomata, which are arranged in staggered arrays, and form appressoria (G, germ tube; A, appressorium; B, branch; bar, 20 μm) (reprinted from *Planta* [148] with permission of the publisher). (**E**) Thigmo differentiation: the differentiation of appressoria by *U. appendiculatus* is induced by topography alone when the fungus is grown on an inert polymer with topographical features that precisely mimic the surface of the host plant (bar, 11.8 μm) (reprinted from *Experimental Mycology* [4] with permission of the publisher).

specific growth responses. Once an experimental system is in place, the next challenge is to define the mechanism of contact sensing at the molecular level. Mammalian cells sense the physical environment through direct contact between the extracellular matrix (ECM) and adhesive proteins that are embedded in the viscoelastic plasma membrane that surrounds the cell. In fungi, the plasma membrane and its embedded proteins are encapsulated by a semirigid polymeric cell wall that separates them from the outside world. It is therefore hard to imagine how these organisms can not only sense contact but also can recognize the size of features in the substrate topography on which they grow. Recent works suggest that the internal organization of the growing fungal cell may play a role in this type of sensing (7). However, for most contact-dependent responses, contact alone is insufficient to induce fungal responses and an additional chemical signal is required, suggesting that contact sensing in fungi constitutes a chemomechanical-coupled system that requires optimal chemical interactions at the fungus-substrate interface in addition to mechanical stimulation. What is more, the developmental stage of the cell enables the fungus to interpret the contact signal within that context. The output, or behaviors, contact recognition elicits in cells is critical to understanding the lifestyle of the fungus, its evolution, and its adaptation. Response behaviors result from the activation of hard-wired signaling pathways that are poised to deliver context-relevant responses. We currently have some understanding of the importance of contact sensing for fungi but can only point to potential mechanisms for mechanosensing at the

molecular level. Thanks to constant advances in technology, we now have a growing catalogue of observed behavioral outputs and the genetic tools with which to test new hypotheses about how contact is sensed. This review will emphasize the importance of contact sensing for fungi and describe the diverse contexts in which specific responses are induced. We discuss the molecular mechanisms that may be involved in generating intracellular signals, with reference to their counterparts in plants, bacteria, and mammalian systems. Some concepts involved in mechanosensing appear to be conserved across these three domains of life.

THE IMPORTANCE OF CONTACT SENSING IN FUNGAL LIFESTYLES

Fungal Contact in the Environment

Contact sensing stands at the threshold of many developmental aspects of the fungal lifestyle and, in most cases, there is a requirement for an additional chemical signal. For other responses, such as the circumnavigation of obstacles, it is not yet known whether chemical signals are involved. Although the molecular mechanism through which contact is sensed has not been elucidated, a number of chemical inputs have been identified that are required for generation of a complete response. The requirement for two stimuli suggests that fungi use a logic gate, where IF there is a physical signal AND there is a relevant chemical signal, THEN a specific response at the cellular and developmental level is generated (Fig. 2).

Initial Contact and Host/Prey Recognition

Many phyto- and entomopathogenic fungi are dispersed as windborne spores where contact with a host heralds the transition from a resting state to growth. Fungal interactions with host plants have been well studied because they constitute the first step in the disease process that can devastate important food crops. To function as a signal that induces new growth and differentiation, the interaction between a spore and its substrate/food source must be quickly stabilized. In plant and insect pathogens, this is achieved through adhesion, but in *A. oligospora*, the nematode-trapping fungus, it is equally well accomplished by the constricting rings formed by capture cells. Both systems require the correct reciprocal chemistry between the fungus and its target (Fig. 3A). In plant and insect pathogens, the spore coat comprises an outer layer of amphipathic proteins called hydrophobins, which have been identified only in filamentous fungi (9, 10). At air-water interfaces, hydrophobins self-assemble to facilitate the growth of aerial structures, but they also mediate adhesion to hydrophobic surfaces through passive, nonspecific interactions (11–13). The chemistry of the outer spore wall therefore establishes adhesion through its compatibility with the hydrophobic epicuticle of the host. Studies of

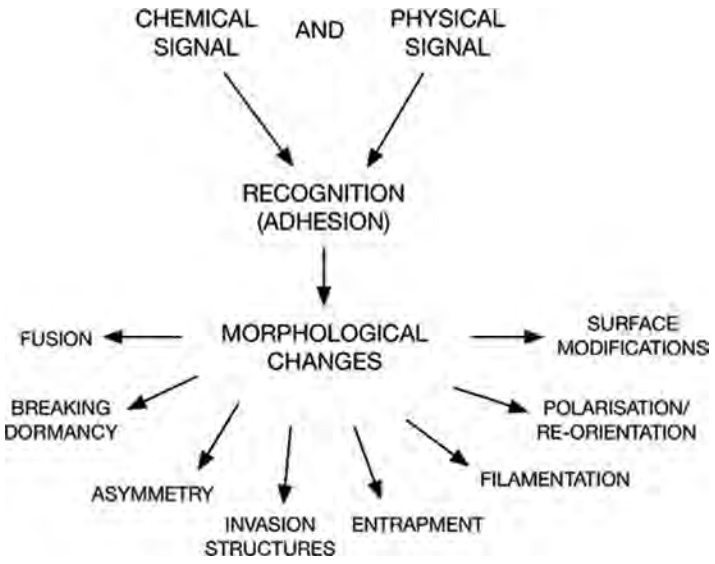


Figure 2 Contact sensing stands at the threshold of a diverse range of morphological and growth transitions relevant to specific fungal lifestyles. Most transitions require a supporting chemical signal for complete induction of a normal response while, in others, this aspect has not been fully investigated.

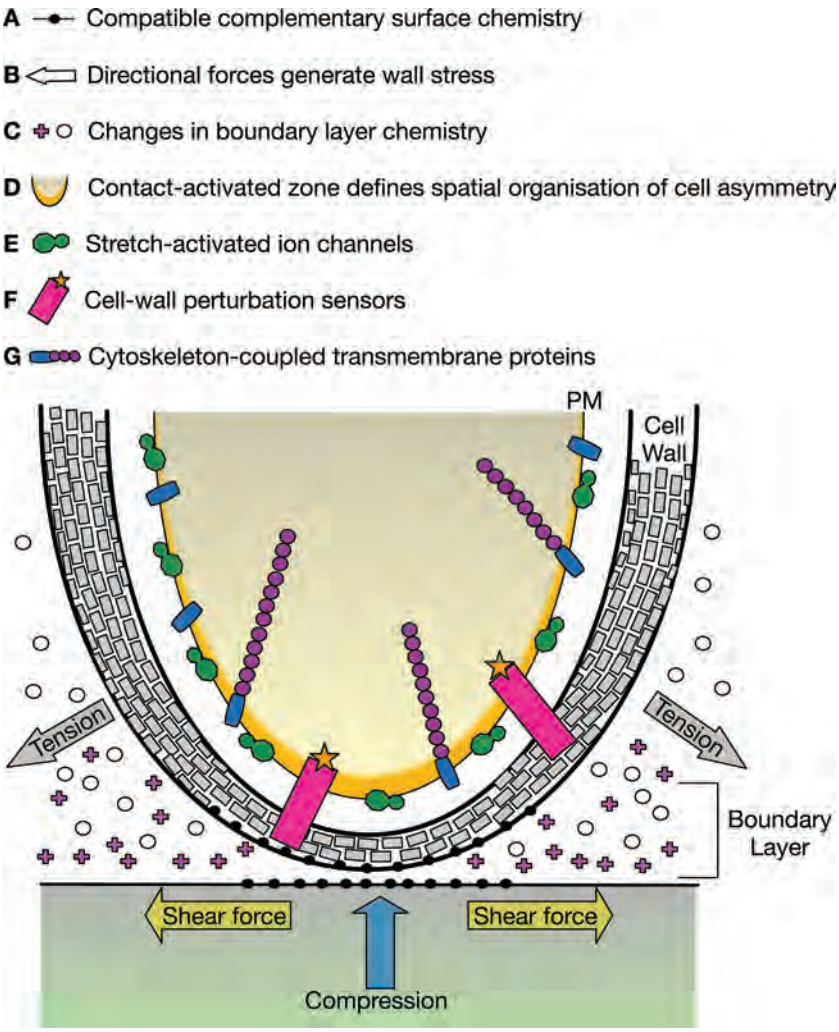


Figure 3 Mechanisms involved in fungal contact resulting in recognition and/or adhesion. (**A**) Compatible surface chemistry between fungal and substrate surface promotes adhesion. (**B**) Directional forces generate cell wall stress. (**C**) Chemical changes sensed within the boundary layer, sometimes as a result of fungal enzyme activity against host exudate. (**D**) Contact-activated zone defines spatial organization of cell asymmetry. (**E**) Stretch-activated ion channels. (**F**) Plasma membrane (PM)-associated cell wall perturbation sensors coupled to intracellular signaling pathways. (**F**) Sensors of cell wall perturbation. (**G**) Cytoskeleton-coupled transmembrane proteins.

hydrophobins show that the arrangement of rodlets differs between fungal species, but they all present an undulating, regularly patterned surface at the plant interface. Some fungi employ additional adhesion mechanisms. The elongated conidia of the rice blast fungus, *Magnaporthe grisea*, attach to surfaces through the release of mucilage from the spore tip after moisture is absorbed, where hydration of the fibrous material causes it to expand and burst through the cell wall (14). There is no evidence that host-specific receptors are recognized by spore surface proteins in plant and insect pathogens, so the avidity of multiple hydrostatic and van der Waals interactions appears to be sufficient to anchor spores to the host (12).

For fungal spores, contact with a suitable substrate is a prerequisite for breaking dormancy, but how is the substrate sensed through the cell wall? The requirement for adhesion suggests a mechanism whereby vibration, wind, or rainfall generates shear stress at the spore-host interface, and this perturbation is somehow transmitted through the cell wall (Fig. 3B). The further requirement for surface hardness by fungal spores supports this idea

because a viscoelastic surface would absorb the energy from external strain, thereby dampening the mechanical signal, potentially to below a threshold level. In addition to shear stress, there is evidence that contact is sensed chemically through changes in the local ionic concentration or chemical species present within the aqueous boundary layer at the interface between plant and fungus (Fig. 3C) (15).

The requirement for two external signals to operate as a simple "AND" logic gate increases the likelihood that a suitable host has been located before resources are committed to the next developmental stage. *Blumeria graminis* and *Uromyces viclai-fabae* carry lipases, cutinases, and esterases on their spore surface so that contact with the cognate host generates specific breakdown products from the long-chain waxes that coat the host leaf surface (16–18). Production of these moieties, or their addition *in vitro*, stimulates spore differentiation so it is likely that they act as a positive-feedback mechanism for host recognition by the fungus (16). While host surface hydrophobicity is required for spore adhesion via fungal hydrophobins, it also facilitates the generation of a chemical signal that is sensed by the fungus. In *M. grisea*, contact with a hydrophobic surface, whether it be a leaf or an "inert" plastic, allows the lipophilic surface-borne inhibitor, pyriculol, to diffuse away from the spore wall, thereby relieving inhibition and permitting germination (19). Chemical signals also play a role in host/prey recognition in the nematode-trapping fungus, *A. oligospora*, but here the logic gate becomes more complicated because the chemical signal is temporally separated from the sensing of contact, and it must come first. Capture cells are induced by the recognition of prey exudates in the environment so that the fungus does not commit these resources unless prey is nearby. There is interspecies variation in the ability of exudate from parasitic nematodes to induce these loop structures but, once generated, capture-cell activation is entirely contact driven and independent of nematode species (20). In all the examples described here, contact involving thigmo- and chemo-sensing drives host/prey recognition. Just as importantly, they also spatially define the zone of the fungal cell surface at which contact has been made (Fig. 3D). This information will determine the direction of asymmetrical cell development in the next stages of gene expression, development, and growth.

Contact Sensing and Plant Pathogenesis

For most plant pathogens, breaching of the tough host-leaf cuticle is a challenge that requires the generation of specialized penetration structures called appressoria. The function of appressoria is to produce a specialized peg that has the capacity to penetrate through the protective cuticle into the tissue below. Appressorial structures vary by fungus and can be dome shaped or appear as swollen hyphal tips, known as infection cushions. Correct orientation of the penetration peg in relation to the host leaf is essential for function and is probably determined by sensing the zone of contact with the host leaf to generate an internal landmark signal that specifies the site of new growth. The majority of biotrophic phytopathogens, including the rice blast fungus, *M. grisea*, form appressoria after a short polarized growth stage on the surface plane of the leaf. The secretion of adhesive material around the zone of contact anchors the base of the appressorium to the host to provide an opposing force against which the penetration peg can push. Sensing of the contact zone also orients the penetration peg toward the underlying leaf and, in *M. grisea*, excludes this site from the process of cell wall melanization and strengthening that is required for the accumulation of solutes and for withstanding the resulting turgor pressure of up to 8 MPa (80 bar) (21). The corn pathogen, *Colletotrichum graminicola*, also uses the zone of contact to define a point adjacent to the leaf surface from which the new germ tube emerges (22). After emergence, the germ tube adopts a flattened morphology against the leaf surface and remains in close contact before the appressorium is generated. Together, these examples demonstrate that surface contact influences cell shape and the direction of polarization. Fungi such as the white clover pathogen, *Cymadothea trifolii*, and the rusts, *Puciinia graminis, Puciinia hordei*, and *Uromyces appendiculatus*, produce exploratory hyphae that search the host leaf to locate epidermal pores called stomata, which are ideal penetration sites. Thigmotropic sensing by hyphal tips appears to be differentially programmed in these fungi to accommodate the host-specific arrangement of stomata within the leaf structure. In the white clover, *Trifolium repens*, stomata are located at epidermal cell boundaries. The hyphae of *C. trifolii* search out these stomata by growing along the meandering depressions between the host cells (Fig. 1C) (23). In contrast, stomata of the maize plant are laid out in a staggered grid, where hyphae of the rust fungi, such as *U. appendiculatus*, grow perpendicularly across parallel host leaf depressions to locate them (Fig. 1E) (23–26). Sensing of the topography of host stomata induces thigmo differentiation, whereby hyphal extension ceases and an appressorium develops over the pore structure. By growing these hyphae on inert plastics featuring ridges of defined heights and spacing, it has been observed

that the exploratory hyphal tips were asymmetrically biased toward the substrate and only responded to topographies that precisely mimicked the stoma dimensions of the host plant (Fig. 1D) (4, 22). The inductive ridge height was optimal at a perpendicular elevation of 0.5 μm, representing 0.06 to 0.1 of the hyphal diameter, and required the presence of two acute angles, suggesting that the hypha was tuned to detect this shape (Hoch et al [25]). Thigmo differentiation, germ tube emergence sites, the orientation of penetration pegs, and the shape of exploratory hyphal tips are morphological responses that are induced by fungal recognition of an external zone of contact with the host plant. This sense of contact then determines the spatial organization of intracellular asymmetry and growth that is required for the next stage of host tissue invasion by phytopathogens.

Contact Sensing and Fungal Mutualism

The antagonistic relationship between fungi and plants is countered by the endophytic and mycorrhizal fungi that support the growth of plants by developing intimate relationships with their host, in general, to their mutual benefit. The processes that establish and maintain these interactions are not well understood because of the paucity of available genetic methods and the challenges involved in imaging the host-fungus interactions in real time *in vivo*. Endophytes such as *Epichloë* and *Neotyphodium* spp. live as hyphae within grasses, permeating through intercellular spaces and extending along the length of the leaf between the host cells (Fig. 4A). Endophytes confer resistance to a variety of external host stresses, such as insect predation and attack by downy mildew, by producing toxic alkaloids and other compounds (27). Endophytes grow synchronously with their host plant, elongating each hyphal compartment as its host's leaves enlarge. It is thought that the tight attachment of hyphae with the host extracellular matrix stimulates hyphal elongation through a sense of stretch (Fig. 4B). Unlike vegetative hyphae that grow by extension at the apex, the hyphae of endophytes extend by incorporating new material into the cell wall, known as intercalary growth (28). As hyphal compartments elongate, new septa are laid down between existing septa instead of forming sequentially as they do during apical growth. The mechanism through which stretch is sensed by endophytes has not yet been established. It seems likely that the shear force along the wall is transmitted to the plasma membrane underneath to signal sites where new wall material is required to relieve the strain. The mechanism of sensing stretch is better characterized in plants, where cells undergo shear stress during the expansion of adjoining cells. The cell wall is linked to the plasma membrane in a punctate manner via structures called Hechtian strands, but these have not been demonstrated in fungi (29). Intercalary growth has been observed previously in the developmental programs of mushrooms, rhizomorphs, water molds, and others (reviewed in reference 30) but the stimulus is unknown in these cases. In endophytes, it is the fungal sensing of contact with the host through adhesion that acts as the important regulatory factor in the rate and spatial control of hyphal extension (28).

The role of contact with an external host is also important in the development of arbuscular and ectomycorrhizal mutualism, whereby formation of close intracellular or extracellular associations with plant roots delivers phosphate and nitrogen, respectively, in exchange for photosynthesis-derived carbon. Location of the host's root apex by the fungus is driven by chemotropic growth toward CO_2 and volatiles present in root exudate (31–33). Recognition and contact with the host drives the development of specialist structures that permit the exchange of nutrients between organisms. Arbuscular mycorrhizae cause an internal rearrangement of the host's cortical cells, which produce a specialist membrane that surrounds the intruding hyphal arbuscules and expresses symbiosis-specific transporters (Fig. 4C) (34). Ectomycorrhizae do not penetrate host cells, instead forming a fungal sheath surrounding the root cortex and a network of hyphae, known as a Hartig net, that surrounds each root cell (Fig. 4D) (35). The genes that drive the development of these fungal structures are being identified, but their functions are as yet unknown. The formation of root-associated structures partially depends on chemical signaling gradients from the host, but it seems likely that these signals are integrated with contact sensing if the fungus is to adapt its own cell wall to fit around the host cell surface, between cortical cell layers, and within host-cell cavities.

Contact Sensing During Cell Fusion

An important feature of the fungal lifestyle is to undergo cell fusion, which can occur with "self" or as "nonself" fusion, which in turn can occur in an intra- or interspecies manner. The process is carried out in two stages, and the pivotal point that marks the end of one stage and commencement of the other is defined by cell-cell contact. During the first stage, the presence of a partner is recognized through secreted chemical signals, and the cells grow chemotropically toward each other along a concentration gradient, a process

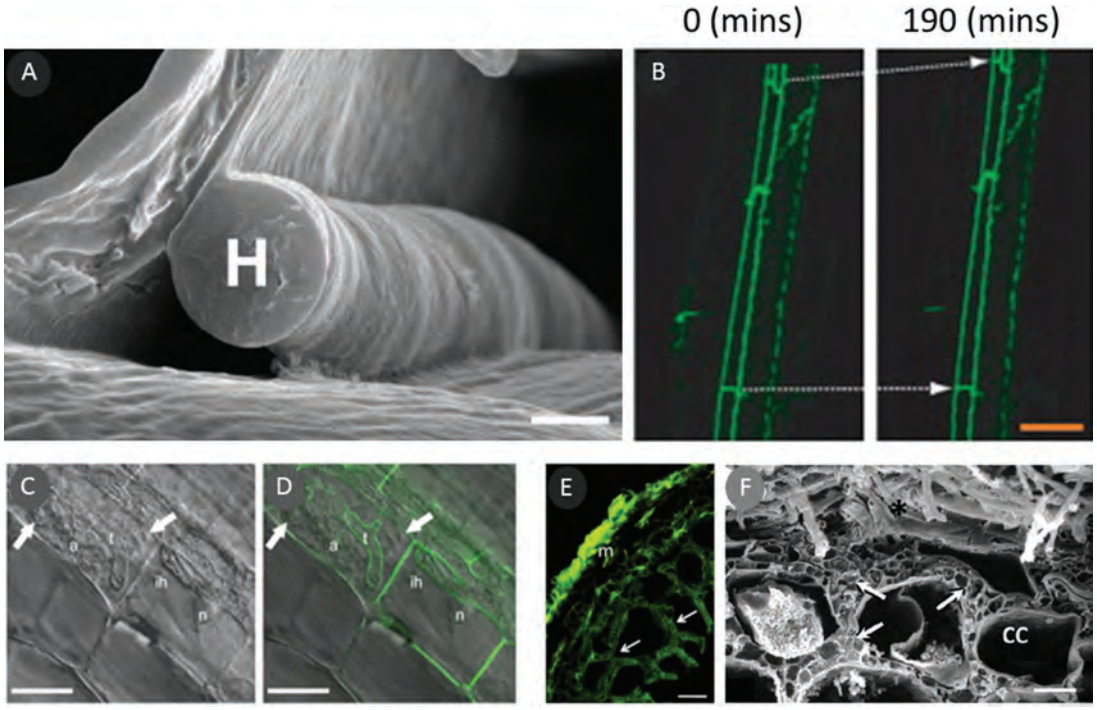


Figure 4 Tight contact between fungus and host during fungal mutualism. (**A**) Host-plant cell growth is thought to be sensed by the endophyte, *Neotyphodium coenophialum*, through its strong adhesion to the host intercellular matrix (H, hypha; bar, 1 μm) (reprinted from *Fungal Genetics and Biology* [28] with permission of the publisher). (**B**) The sense of stretch induces intercalary growth of the endophyte, *Epichloë fesctucae*, so that it elongates at the same rate as the host. Arrows indicate the lateral branches that have moved further apart as a result of intercalary growth (bar, 100 μm) (reprinted from *Fungal Genetics and Biology* [28] with permission of the publisher). (**C, D**) Arbuscular mycorrhizae form intruding hyphal arbuscules within the root cells of *Medicago truncatula*. Host GFP-Mtcp1 localizes to the cell plasma membrane and its derivative that surrounds the arbuscule trunk, but is not expressed in host membrane surrounding arbuscule branches (arrows), which is thought to be involved in nutrient exchange (a, arbuscules; t, arbuscule trunk; ih, intracellular hyphae; n, nucleus; bar, 20 μm) (reprinted from *Plant Physiology* [34] with permission of the publisher). (**E**) *Hebeloma cylindrosporum* ectomycorrhizae, labeled with wheat germ agglutinin-fluorescein isothiocyanate, grow as a dense outer fungal sheath or mantle surrounding the "Hartig net" (arrows) of hyphae growing between outer cortical cells of hazelnut, *Tuber melanosporum* (m, fungal mantle; bar, 15 μm) (reprinted from *Frontiers in Plant Science* [149] with permission of the publisher). (**F**) *H. cylindrosporum* forms a fungal sheath surrounding the Hartig net (arrows) of hyphae that intercalate between the cortical root cells of *Pinus pinaster* (CC, cortical cells; black asterisk, fungal sheath; bar, 10 μm) (reprinted from *Molecular Plant-Microbe Interactions* [35] with permission of the publisher).

that has been termed "homing." Contact between the cells marks the spatial and temporal point at which growth ceases and the second phase commences, during which the cell walls are dissolved and the plasma membranes are reorganized to allow the mixing of cytoplasmic material. Cell fusion generally occurs between specialist fungal structures, and the response to cell-cell contact is specified by this developmental context. The best characterized example of non-self-cell fusion is the mating response in *Saccharomyces cerevisiae*, where cells of opposite mating types form blunted, polarized mating projections called "shmoos." The chemical signaling molecules, **a** and α pheromone (peptides), have been identified (36, 37) and the shmoo tips grow chemotropically toward each other until contact is made, whereupon the cell walls dissolve, the plasma membranes fuse, and nuclear fusion takes place (Fig. 5B). The mechanism for sensing contact in this process remains elusive and it has been generally understudied, given its pivotal role.

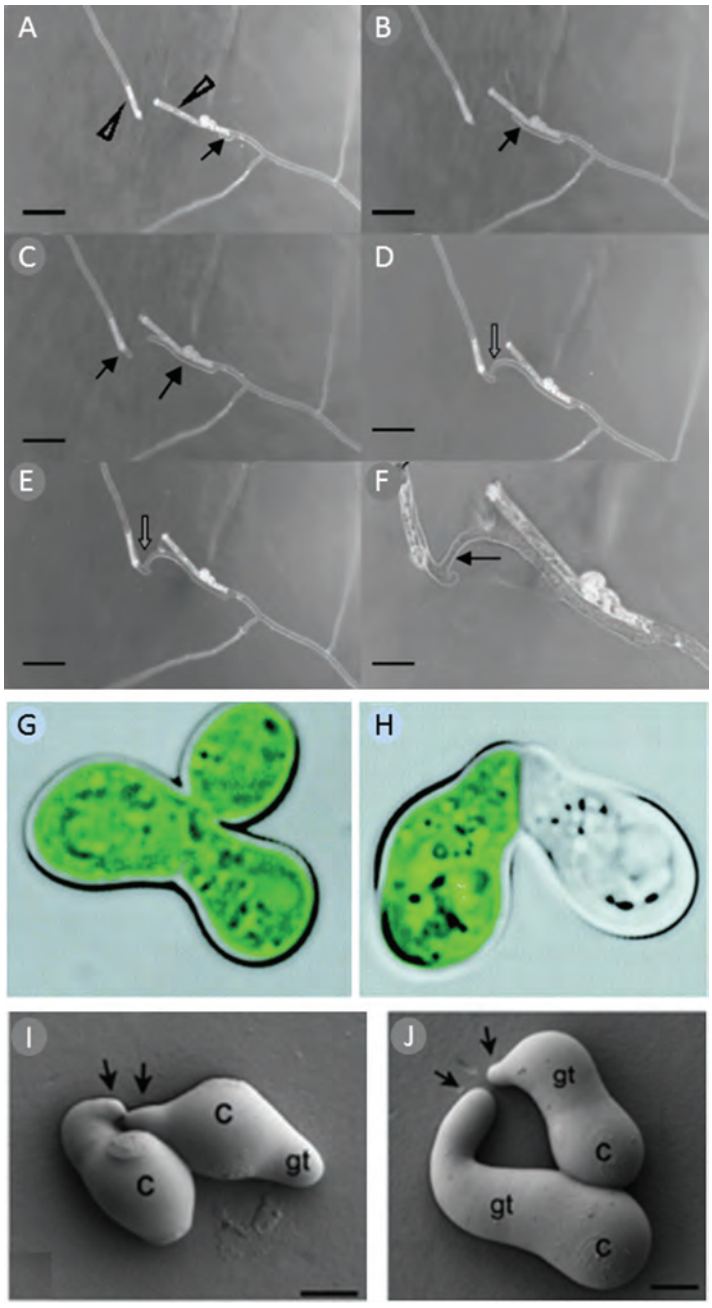


Figure 5 Cell-cell contact marks the developmental tipping point between "homing" and fusion. (**A to F**) Healing process between the ends of a severed hypha in mycelia of the arbuscular mycorrhiza, *Gigaspora margarita*. (**B, C**) Severed hyphal compartments (open arrows) are sealed off by the formation of new septa. New hyphae emerge from behind the septa and grow chemotropically toward each other (closed arrows). (**D to F**) Hyphal wall contact and fusion (open arrows) permits cytoplasmic flow to be reestablished (open arrowhead). (Bars, 80 μm) (reprinted from *New Phytologist* [39] with permission of the publisher). (**G**) Mating cells of yeast *S. cerevisiae*, where the shmoos of two wild-type cells expressing soluble GFP have fused by degrading the cell wall and fusing the plasma membrane. A daughter cell has emerged (top). (**H**) In the *prm1*Δ null mutant, which lacks a multispanning transmembrane protein, shmoo-shmoo contact triggered cell wall degradation but not membrane fusion, stalling the mating process (reprinted from the *Journal of Cell Biology* [150] with permission of the publisher). (**I**) Conidial anastomosis tubes (CATs) emerge from conidia of *N. crassa* and "home" toward each other (c, conidia; gt, germ tube; bar, 6 μm). (**J**) CATs emerge from germ tubes prior to contact and fusion (bar, 5 μm) (reprinted from *FEMS Microbiology Letters* [44] with permission of the publisher).

Filamentous fungi generate spreading branched networks that form interconnections, called anastomoses, between vegetative hyphae or between specialized fusion filaments. In soil fungi that scavenge for resources in heterogeneous environments, anastomosis improves colony resilience by creating efficient routings for the distribution of nutrients (38). The imperative to maintain colony integrity is evidenced by the arbuscular mycorrhizae, *Gigasporaceae* and *Glomeracea*, which use anastomosis in an elaborate wound-healing process that rejoins the two ends of a severed hypha (Fig. 5A). This is achieved by first forming a septum either side of the wound to stem cytoplasmic leakage. A new branch then emerges from each sealed compartment and grows toward the other to fuse, thus restoring normal hypha function and nutrient flow (39). Mycorrhizal fungi undergo intraspecies anastomosis between extraradical mycelia that are already symbiotically associated with plant roots and compatible germlings that are not (40). Not only does this maximize nutrient scavenging for the host plant, but it benefits the fungus in two ways. First, it enhances species survival by providing germlings with host-derived carbon before they have formed their own mutualistic association and, second, it improves species fitness through the exchange of nuclear material (41).

Intrahyphal fusion that relies on the formation of a lateral hyphal peg is a mechanism used for the formation of clamps in basidiomycetes such as *Coprinus cinerea* and *Schizophyllum commune* and for trap formation in the nematode-trapping fungus, *A. oligospora*. Hyphal clamps ensure the correct distribution of dikaryotic nuclei through the formation of a lateral peg that acts as a homing device for the tip of a hook-shaped cell that protrudes just ahead of it. After formation of a septum that divides the hypha between the two structures, the hooked clamp cell fuses with the peg to deliver a daughter nucleus to join its genetically different partner (42). Hyphal trap formation involves anastomosis between a lateral hyphal peg and the distal end of the three specialized trap cells in order to form a complete loop. An exchange of cytoplasm takes place before a septum is formed at the site of fusion and the trap is complete (43). The lateral peg mechanism is consistent with the overall theme of fungal fusion, whereby sensing, homing, and fusion require highly defined apical zones in which the function-specific molecular machinery is confined. The intriguing nature of the signaling systems during anastomosis has been investigated in the homing of conidial anastomosis tubes (CATs). Twenty-one genera and 73 species of filamentous fungi, primarily *Aspergillus* spp. and *Penicillium* spp., form CATs, which are short, thin hyphae that home toward each other and fuse (Fig. 5C) (44). The nature of the extracellular signaling molecules and their receptors remains to be identified but, in *Neurospora crassa*, CAT signaling involves the oscillatory antiphase recruitment of the proteins, MAK-2 and SO, to the two homing tips in a process termed the "ping-pong" mechanism of call and response (45, 46). Although CATs form between various types of conidia of the same species, they also emerge from mature hyphae, and even interspecies fusion has been observed, which can produce stable heterokaryons (47). Since nutrient limitation increases the frequency of germling fusion, and clonal, rather than sexual, replication dominates among filamentous fungi, nutrient sharing and the exchange of genetic material appear to be the most common benefits to fungi in the evolution of cell-fusion mechanisms (48, 49). As with all the examples of fungal fusion highlighted here, recognition that tip contact has been achieved is an essential component of the process.

Fungal Contact with the Human Host

Every day, humans inhale hundreds of airborne spores produced by environmental fungi, yet very few fungal species cause infections, and then mostly only in patients with immune deficiencies or specific underlying medical conditions, such as HIV infection. Most environmental fungi are unable to survive the relatively high body temperature of mammals and they are efficiently cleared by the host immune system. The inhaled spores of *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Pneumocystis jirovecii* comprise three of the top four causes of fatal fungal infection, with mortality rates of 20 to 90% (50). Other fungi that afflict the human population are transmitted through direct contact with a colonized surface. These include *C. albicans*, a commensal yeast of the mucosa that is primarily transmitted from passage through the birth canal (51), the dermatophytes of keratinized skin such as *Trichophyton rubrum*, which transfers through surface contact and causes "athlete's foot," and dermatophytes of the glabrous skin, such as the zoonotic fungus, *Microsporum canis*, which produces the circular lesions known as "ringworm" and is transmitted through person-to-person contact or between people and animals (52).

For the inhaled spores of environmental fungi, the dominant signal for contact with a human host is likely to be the sustained rise in temperature and ambient moisture. Human surfaces present an extremely complex landscape of chemistries, are highly variable by

body site, and, in general, are not amenable to *in vivo* study so our understanding of contact-dependent interactions requires reductionist methods. Studies of human pathogenic fungi that are acquired by direct transmission often have a complex fibrillar outer surface composed of glycoproteins, unlike the smoother hydrophobin coat of conidia from environmental fungi (53, 54). The advantage to fungi of glycoprotein fibrils is that they can present diverse surface chemistries simultaneously to maximize the likelihood of achieving adhesive interactions with host surfaces. The fibrils of the dermatophytes *T. rubrum* and *Trichophyton mentagrophytes* include lectin-like adhesins that bind host-surface carbohydrates, while *C. albicans* expresses a large number of glycoprotein adhesins that enable cells to bind to RGD moieties in extracellular matrix proteins (e.g., collagens and fibronectin), surface carbohydrates, specific receptor proteins, and implanted medical plastics (55–60). Of particular note is the *C. albicans* hypha-specific cell wall protein, Hwp1, which is covalently cross-linked to keratinized cells through its N-terminal domain by host transglutaminase (58). Whether this linkage acts as a signal through the Hwp1 C-terminal of Hwp1, which is covalently bound to cell wall β-glucan, is not known. To date, although numerous types of interaction take place between the host and extracellular fungal fibrils, none is known to directly act as part of a trans-cell wall protein signaling pathway to indicate that host binding has occurred. As with phytopathogens, contact is more likely to be sensed by general cell wall perturbation across the zone of adhesion that activates stretch sensors in the underlying plasma membrane. A role for chemical sensing of the host environment to support contact-induced signals could occur in the form of nutrients that are released from the host. *C. albicans*, for example, carries surface-mounted aspartyl proteases that function at different pH optima. Their activity may indicate not only that adhesion has been achieved, but also identify the site of adhesion by virtue of the chemical profile of host-related products that is generated (61).

Contact with a compatible surface that facilitates adhesion is the critical first step in host colonization and a signal for the initiation of new fungal cell growth. In filamentous pathogens, this is usually accompanied by cell asymmetry, polarization, and differentiation before transferring from surface growth to embedded growth in a tissue matrix. The outer cornified epidermal layer to which dermatophytes adhere is subject to continuous abrasion and cell loss. To counter this, the arthroconidia of *T. rubrum* and some *Malassezia* species produce filamentous hyphae that undergo anastomosis and intercalate between the keratinized cell layers to form a subcutaneous mycelial network that can withstand the loss of the outer epidermal cells. In *T. rubrum*, HexA, the primary component of the Woronin body that seals off hyphal compartments on wounding, is upregulated during growth on keratin, suggesting that this is important for the integrity of the mycelium in the elastic milieu of the epidermis (62, 63). *C. albicans* produces hyphae when immune surveillance is locally deficient or is compromised because of medical interventions. Hypha production may be a stress response that does not occur in the commensal environment of the gastrointestinal tract, where there is immune cell tolerance and sufficient access to nutrients. Upon adhesion to the oral or vaginal mucosa, hyphae invade the underlying epithelial layers, causing localized inflammation. Serum is a powerful inducer of morphogenesis and yeast cells that access the bloodstream through surgery or by nosocomial transmission soon generate penetrative hyphae that facilitate escape of the fungus into underlying solid organs. *C. albicans* yeast readily adheres to medical plastics, where hyphae germinate to form resilient three-dimensional biofilm networks, often containing other *Candida* species and bacteria (64). Adhered hyphae can apply sufficient force to penetrate soft medical silicones such as voice prostheses, causing the material to swell and stiffen, leading to failure of the device (65). In the lung, *Aspergillus* conidia swell, fragmenting their hydrophobin coat prior to hypha formation, which results in tissue distribution patterns that are dependent on the underlying disease and immune status of the host. As seen for plant pathogens, contact with a compatible host facilitates cell adhesion and the induction of downstream developmental pathways. In some host-fungus relationships, this leads directly to disease and, in others, to compatible responses, immune tolerance, and fungal commensalism. In all cases, the fungal sense of contact is inextricably linked to mechanisms that maintain the association of the fungus with the host.

THE MOLECULAR BASIS FOR CONTACT-INDUCED DIRECTIONAL GROWTH RESPONSES

Despite its important role in the fungal lifestyle, the molecular basis for contact sensing by fungi is unknown. In contrast, the downstream signaling pathways for the developmental phases triggered by the sensing of contact are reasonably well defined in a number of fungi. In part, this imbalance is due to the difficulties of study-

ing contact at the level of individual cells, especially resting conidia, and differentiating between defective input versus output pathways. Progress has recently been made using growing hyphae of *C. albicans*, in which there is an increasing availability of molecular tools that can be exploited in imaging systems using microfabricated surfaces and fluorescence live-cell imaging. These approaches are helping to characterize contact-dependent responses that can be disrupted by gene deletion or mutation. Studies of actively growing hyphae showed the effect of contact events with topographical features that perturb the direction of growth and induce specific growth responses. Over the decades, imaging has consistently shown that hyphae growing on a surface adopt an asymmetric tip morphology with the tip pressed to the substrate (66). This is particularly striking in *Uromyces* hyphae as they explore the host leaf surface in search of the small topographical cues indicative of stomatal penetration sites (Fig. 1D). *C. albicans* hyphae also become asymmetric during growth on surface, where they can respond to topographical ridges of less than half the hyphal diameter by reorienting their growth axis to follow the contour (67). The basis for this morphology and the role it plays in sensing surface features has been partially elucidated (7). Green fluorescent protein (GFP) labeling of cell polarity proteins including myosin light chain 1 (Mlc1) and Spa2, markers for the Spitzenkörper and polarisome, respectively, demonstrated that the molecular complexes involved in polarized growth were also asymmetrically skewed toward the point of hyphal tip contact (Fig. 6A). Thus both the tip shape and the intracellular polarity complexes within are biased toward the substrate. When growing hyphae encountered channels and ridges, the Spitzenkörper translocated across the hyphal apex to the new point of contact (7). This asymmetric, contact-mediated positioning was crucial for generating thigmotropic behaviors in *C. albicans*, which included contour-following, gap penetration, and the maintenance of an overall growth trajectory, even after circumnavigating small obstacles (Fig. 6A through D). Deletion of the internal landmark GTPase, Rsr1/Bud1, abolished tip asymmetry and affected the stable localization of the Spitzenkörper and the ability of hyphae to steer normally in response to obstacles (Fig. 6E through G). These findings suggest that Rsr1 mediates outside-in signaling that locates the growth zone at the point of contact, and, in this way, very small changes in topography can be sensed. The upstream signal that localizes Rsr1 activity at the point of contact in *C. albicans* is not known, but, in *S. cerevisiae*, Rsr1 is localized to the

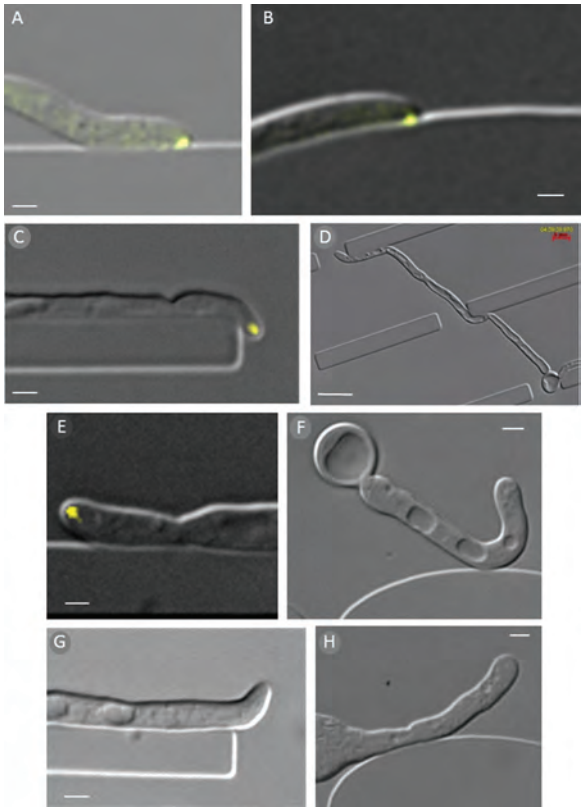


Figure 6 Contact-induced hyphal tip responses in *C. albicans* (7). (**A**) Tip and Spitzenkörper asymmetry induced by contact of a hyphal tip with an obstacle in control cells expressing Mlc1-YFP (bar, 2 μm). (**B, C, and D**) Hyphae exhibit contour following, gap penetration, and trajectory maintenance. (B, C, bars, 2 μm; D, bar, 10 μm). (**E to H**) In hyphae of the *rsr1*Δ mutant, the Spitzenkörper tends to be centrally positioned with the apex, hyphal tips do not become asymmetrical on contact or respond normally to substrate topography by following contours or penetrating gaps (bars, 2 μm) (reprinted from *Cellular Microbiology* [7] with permission of the publisher).

poles of the cell by Rax2, a single-pass transmembrane protein that binds the Rsr1 activator, Bud5. Deletion of Rax2 in *C. albicans* hyphae did not severely affect contact-dependent tip reorientation responses, so does not appear to act as the sensor that localizes Rsr1 activity during growth on a surface (68). Deletion of *RSR1* in *C. albicans* severely attenuated virulence and the ability of hyphae to penetrate and damage host cell layers *in vitro* (5, 6). Together with observations from *Uromyces*, it would appear that the contact-induced asymmetrical organization of the growing hyphal tip plays an important role in host invasion by a range of plant and human fungal pathogens. In *C. albicans*, it was observed that perpendicular contact of a hyphal tip with an obstacle temporarily stalled tip extension until

a new growth direction was determined. During this period, the fungus exerted a force of approximately 8.7 μNagainst the obstacle before reorienting, which theoretically is sufficient to penetrate a human cell plasma membrane without the use of enzymes (7). However, the force generated was influenced by carbon source. Growth of cells in lactate at concentrations reflecting physiological levels at body sites such as the urogenital and gastrointestinal tracts generated a thinner hyphal cell wall with a higher Young's modulus (69, 70). Lactate-grown hyphae reoriented more readily on contact and applied approximately half the force of glucose-grown cells (D. D. Thomson and A. C. Brand, unpublished data). This indicates that nutrient availability influences contact-induced hyphal growth behavior by altering cell wall properties, and this may be relevant to disease development by *C. albicans* at specific body sites. This observation also poses the question as to how hyphae with a thin, stiff cell wall respond more quickly to contact than cells with a thick, soft cell wall.

Potential Mechanosensing Systems in Fungi

To date, no protein or complex has been definitively shown to be a contact-induced mechanosensor in fungi, although there is good evidence that calcium ion (Ca^{2+}) influx is important for signal propagation (71–73). The importance of a sense of touch in fungal decision making suggests that a definable molecular mechanism does exist in fungi. It may be a discrete system that awaits discovery, or it may co-opt other signaling pathway sensors where signal integration indicates that contact has occurred. Here, we speculate on the systems that could be involved in contact sensing, starting with the most likely candidates that have either been linked to contact-induced behavior in fungi, or they bear similarities to mechanosensing systems in mammalian, bacterial, or plant cells. We then discuss other signaling proteins that might be predicted to act as sensors because they function in fungal cell wall integrity surveillance, maintenance, and remodeling processes. Last, we move to the possibility that fungi use as-yet-unidentified functional analogues of mechanosensing systems in animals. The proteins and their downstream signaling pathways discussed below are shown in Fig. 7.

Mechanosensing Ion Channels

Ion channels that are activated by membrane stretch have been identified in all kingdoms of life. They have been best characterized in bacteria, where the MscS (small conductance) and MscL (large conductance) channels are required for resistance to hypo-osmotic stress by releasing solutes from the cell (74, 75). The precise gating mechanisms of these multimeric protein complexes are still unknown but are thought to involve force transmitted from the lipid bilayer, differential partition of components within the bilayer, and the nanoscale properties of water within the pore (76–78). In whole cells, membrane interactions with the cytoskeleton and the presence of a cell wall also influence the pressure required to open mechanosensing channels (79, 80). Mechanosensing channel activity similar to that in bacteria has been identified in some fungi by using patch clamping (Fig. 3E). *S. cerevisiae*, *C. albicans*, *N. crassa, U. appendiculatus*, and *Schizosaccharomyces pombe* exhibited conductance from one or two channels using this method, and in *S. cerevisiae* it was confirmed that the mechanosensing channel was regulated by membrane tension and not by outward pressure (67, 79, 81–83). In *S. pombe*, two channels, Msy1 and 2, localized to the endoplasmic reticulum and protected the vacuole and nucleus from hypo-osmotic shock (84). Single, nonselective channels were observed in *S. cerevisiae* and *U. appendiculatus* and were calculated to occur at a density of 4–6 and 2 channels per μm^2, respectively (79, 82). Whether this channel density is sufficient to sense membrane tension in the tip of *U. appendiculatus* during contact with inductive ridged topographies is not known, but localization has not been determined and channels may cluster at the growing apex. Alternatively, these channels may be sparsely dispersed but transmit a potent signal. Channels in both fungi were found to be relatively selective for entry by calcium ions. Ca^{2+} is a signaling molecule involved in many stress-related pathways so the resting cytoplasmic calcium concentration is maintained at levels of ~0.1 to 0.2 μM in fungi, establishing a steep electrochemical gradient across the plasma membrane. It was calculated that only two stretch-activated Mid1 channels in *S. cerevisiae* would need to open for 0.59 s to accumulate sufficient cytoplasmic Ca^{2+} to activate calmodulin and the phosphokinase C pathway, demonstrating how few channels may be required for localized contact sensing, signaling, and cell development (85).

The role of Ca^{2+}-influx in mechanosensing was established through studies of the *S. cerevisiae* protein, Mid1 (Mating-induced death 1) (86, 87). Deletion of *MID1* reduced calcium uptake and stalled the mating process after shmoo formation, ultimately leading to cell death. The phenotype could be rescued by the addition of exogenous $CaCl_2$. When expressed in Chinese hamster ovary cells, ScMid1 conferred a response to mechanical stress by inducing a rise in intracellular

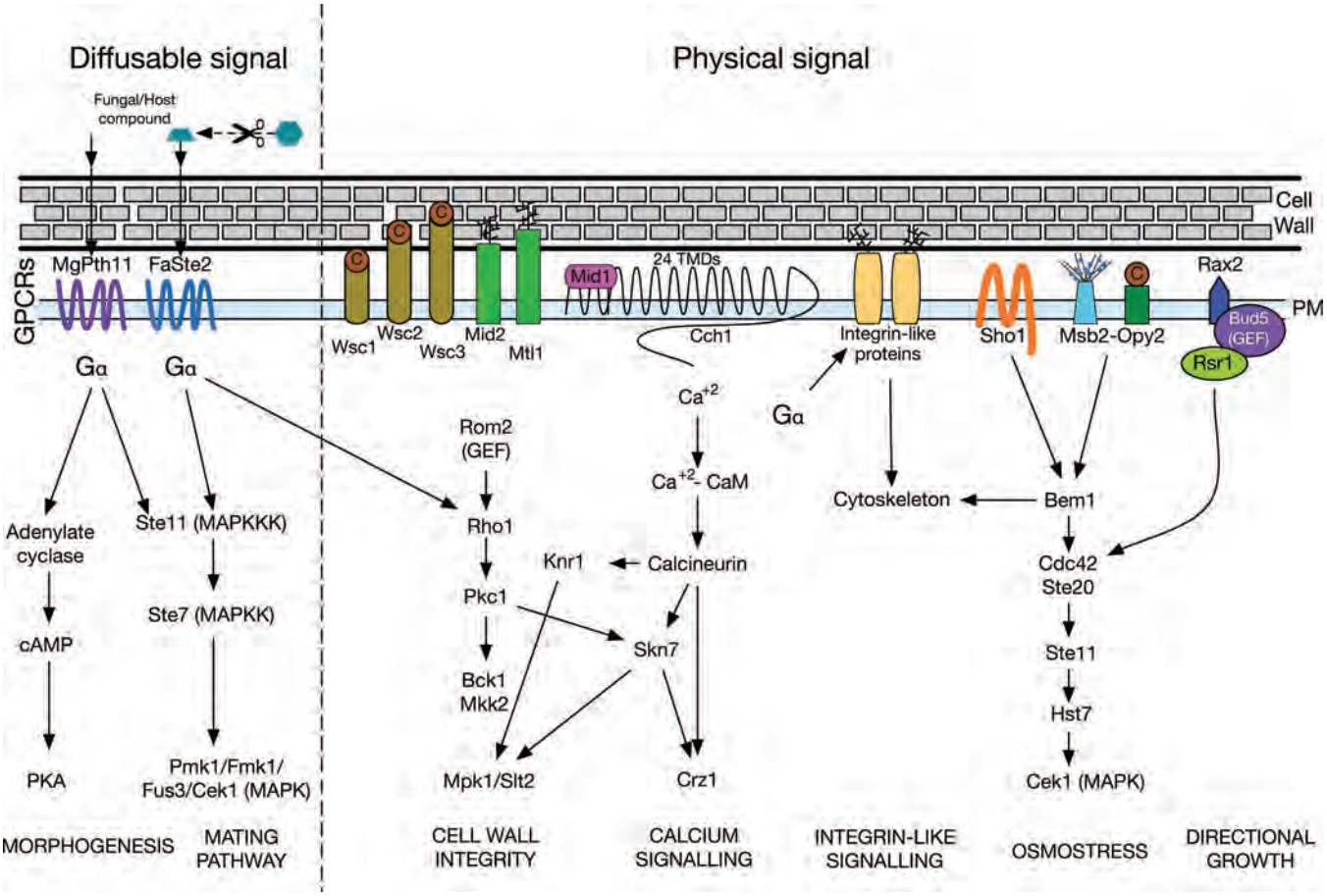


Figure 7 Sensing and signaling pathways that have the potential to be involved in mechanosensing in fungi. For details and references, see the text. PM, plasma membrane.

Ca^{2+}, suggesting it was a stretch-activated Ca^{2+} channel (88). In *C. albicans*, Mid1 is involved in Ca^{2+} uptake and was required for normal contact-dependent hyphal tip reorientation (72). In other fungi, Mid1 was generally found to be involved in calcium homeostasis but other deletion phenotypes differed from *S. cerevisiae*. In *N. crassa*, turgor pressure and growth rate were reduced but mating was unaffected, and the *mid1*Δ mutant in *A. fumigatus* was hypervirulent (89–92). ScMid1 was predicted to contain four transmembrane domains and localized to the endoplasmic reticulum and the plasma membrane (85). The identification of ScCch1, a homolog of the α-subunit of mammalian voltage-gated L-type Ca^{2+} channel (VGCC), ultimately led to the view that Mid1 was not a channel but a subunit, and potentially a regulator, of the Cch1 high-affinity calcium uptake system (HACs) (93–96). In *C. albicans*, where Cch1 has high similarity in its pore-forming and voltage-sensing domains to human CaV1.2 voltage-gated Ca^{2+} channels, the Mid1 peptide sequence is cysteine rich and contains a potential ω-site at Ser^{537}, suggesting that it is glycosylphosphatidylinositol (GPI) anchored and the bulk of the protein is extracellular. This overall topology is similar to the α2δ regulatory subunit of mammalian VGCCs, which also contain a cysteine-rich C terminus and are GPI anchored (97). Other Mid1-like proteins with cysteine-rich C terminals but no GPI anchors have been identified in animals, where they control the activation threshold of cation channels (98). In animals, VGCCs are also regulated by γ subunits. Several proteins have been identified as potential homologs of γ subunits in *S. cerevisiae* but only two, Ecm7 and Fig1, have been examined by gene deletion. Fig1 is upregulated in *S. cerevisiae* and *C. albicans* during mating, where it localizes to membranes that are destined to fuse (99, 100). Its deletion in *S. cerevisiae* did not inhibit fusion but produced aberrant zygotes with incomplete clearance of membrane or wall at the fusion site (101). Thus, Ca^{2+} is again linked with a cellular process that

involves contact-dependent responses, but the sensing mechanism that triggers Ca^{2+} influx into the cytoplasm is not known.

Cell Wall Integrity Pathway Sensor

Five single-pass transmembrane proteins in *S. cerevisiae* are specifically involved in sensing perturbation of the cell wall by virtue of N-terminal head groups that are embedded within the matrix of the wall, making them candidate mechanosensors that could mediate contact-dependent responses (Fig. 3F). The head groups of Mid2 and Mtl1 are large, branched *N*-glycans, while those of Wsc1, 2, and 3 comprise eight cysteine residues in cysteine-rich domains (CRDs) through which they are thought to cluster (reviewed in reference 102). Representatives of the two protein families are found in *Candida glabrata*, *A. fumigatus*, and *Cryptococcus neoformans* (reviewed in reference 103). Compounds that bind and disrupt hydrogen bonding between the structural cell wall β-glucan or chitin polymers, such as Congo red and calcofluor white, or echinochandin antifungals that inhibit glucan synthase, activate the cell wall integrity (CWI) signaling pathway via Rho through the Slt2/Mpk1 mitogen-activated protein (MAP) kinase cascade to upregulate cell wall biosynthesis. The computed live cell extension length of CWI sensors varies from 61 nm (Mid2) to 106 nm (Wsc3), where the cell wall in *S. cerevisiae* has been measured at 102 nm. These CWI proteins may partially span the cell wall in *S. cerevisiae* but seem too short to act as direct sensors for external cell wall contact. In *C. albicans*, cell wall thickness varies by at least 50% in a carbon-source-dependent manner (70) so Wsc1 and Mid2 family proteins, with their transmembrane domain, cell wall-embedded head group, and potential extracellular reach, could constitute functional mechanosensors. In support of this idea, in *C. albicans*, although not in *S. cerevisiae*, growth on a surface activates Mpk1, the MAP kinase of the CWI signaling cascade (104). However, the nano-spring structure of Wsc1 that enables it to stretch (105), CWI activation through treatments that weaken the cell wall, and the role in sensing hypo-osmotic stress (106) suggest that Wsc1/Mid2 function is to sense outward cell wall tension rather than contact-induced compression. Cell wall tension indeed occurs during some contact events, for example, at either side of the hyphal tip during perpendicular contact with an obstacle (7). However, an argument against the involvement of Wsc1/Mid2 in contact sensing is that activation of the CWI pathway leads to Rho-mediated depolarization of the cytoskeleton and redistribution of the wall biosynthesis machinery (107), which is inconsistent with the observation that most contact events in fungi induce the development, or retention, of cell asymmetry.

Sensors of Osmotic Stress

Hyperosmotic shock, such as exposure to 1 M NaCl, causes *C. albicans* yeast cells to shrink to half their volume before recovering via the high-osmolarity glycerol (HOG) signaling pathway, which comparative genomics has shown is generally well conserved across a sample of 38 fungi (70, 108). The sensors that trigger the response are localized in the plasma membrane and activate Hog1 through the histidine kinase, Sln1, or through two Sho1-based pathways, both of which signal through Cdc42 and Ste20. Proteins of the *SHO1*-related pathways (Msb2, Opy2, and Hkr1) form large, planar oligomers where the mucins, Msb2 and Hkr1, which are highly *O*-glycoslyated, and Opy2, with its cysteine-rich domain, are activated by conformational change (109, 110). If cell contact is signaled by cell wall changes that result in mechanical perturbation of the cell membrane, these structures could potentially act as mechanosensors. The Msb2 mucin-like protein is a particularly intriguing protein because it is strongly linked to fungal pathogenesis through extracellular and intracellular functions. In *C. albicans*, all the functions of Msb2 rely on its cleavage to shed the large glycosylated extracellular domain (111). In mammalian cells, mucin cleavage is autoproteolytic through the activity of an internal 120-amino-acid SEA domain (112). This domain is lacking in *C. albicans* Msb2 and it is thought that Msb2 cleavage occurs through the activity of a dedicated protease, placing Msb2 downstream of the sensor protein. Release of the glycodomain protects the fungus by binding to host antimicrobial peptides. In *C. albicans*, Msb2 is involved in the detection of defective surface protein glycosylation and activates the CWI pathway (111), as it does in *Fusarium oxysporum* (113–115). In *Ustilago maydis* and *M. grisea*, Msb2 is required for detection of hydrophobic surfaces and cutin monomers, while in *Botrytis cinerea* it regulates the secretion of proteins (116–118). Given that contact sensing in fungi generally induces cell asymmetry, the strong links between Sho1-Msb2-Opy2 and their downstream effector, Cdc42, which also regulates cell polarity, hint that this signaling pathway may play a role in contact-induced responses.

G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are seven transmembrane proteins that are coupled to the intracellular heterotrimeric subunits Gα, Gβ, and Gγ. GPCRs ex-

change GTP for GDP on Gα, which dissociates from Gβγ so that all three subunits can participate in multiple downstream signaling pathways. Six classes of GPCR have been classified in fungi where they are primarily involved in pheromone and nutrient detection (119). However, the nature of the binding ligand in fungal GPCR homologs varies by species and the downstream pathways are differentially wired. For example, Gpr1, the glucose-sensing GPCR in *S. cerevisiae*, appears to be activated by methionine in *C. albicans* where glucose is instead sensed via Cdc25 or Ras (120). Likewise, in *F. oxysporum*, plant exudate is sensed via the GPCR, Ste2, a functional homolog of the pheromone receptor in *S. cerevisiae* (121). Importantly, activated Gα subunits stimulate generation of the small cytoplasmic signaling molecules, cAMP and Ca^{2+}, which activate protein kinase A (PKA) and calmodulin, respectively. Activation of the PKA pathway is involved in cell morphogenesis and differentiation in many fungi, including budding growth in *U. maydis*, hypha induction in *C. albicans*, and appressorium formation in *M. grisea* (122–126). In the nematode-trapping fungus *Arthrobotrys dactyloides*, pressure applied by the nematode on the inner surface of the trap cells is thought to activate heterotrimeric G proteins without a chemical ligand (127). Experiments using calcium ionophores and a calmodulin antagonist in this organism suggested that the activated Gα subunit increased cytoplasmic calcium and activated the calmodulin pathway to open aquaporin water channels and inflate the capture cells. Although the GPCR has not been identified in this fungus, the assumption is that GPCRs can respond to pressure that is exerted against the cell wall and could therefore be mechanosensors in some species. In support of this, Pth11, a novel GPCR in *M. grisea*, is thought to sense inductive surfaces because deletion of the gene resulted in an 85% reduction in the number of appressoria produced, although those that were produced were functional (128, 129). The phenotype was similar to that observed when spores were exposed to noninductive surfaces, suggesting that Pth11 could act as a sensor at the top of the signaling pathway. In mammalian cells, GPCRs can function in a ligand-independent manner to sense membrane perturbation. In endothelial cells, studies using conformation-sensitive fluorescence resonance energy transfer showed that GPCRs are activated by the mechanical effects of shear flow (130), and, in neutrophils, shear stress is sensed by the formyl peptide receptor GPCR to alter cell shape and reduce pseudopod projection (131). However, in mammalian cells, GPCRs are exposed on the cell surface. In fungi, GPCRs are exposed instead to the inner surface of the cell wall, so, although their activity is certainly required for contact-induced responses, it remains to be determined whether they do indeed sense contact, perhaps via membrane perturbation, or whether they act as the receptor for the additional chemical signal that is required in the logic gate to induce a new developmental stage.

Integrin-Like Proteins

An important mechanosensing system in mammalian cells comprises single-pass transmembrane proteins called integrins, which function in focal complexes that link the cell-derived ECM to the cytoskeleton via the intracellular scaffolding protein paxillin. Integrins are type I transmembrane proteins that bind as heterodimers to secreted ECM proteins, such as fibronectin and vitronectin, to sense substrate stiffness and provide traction during cell migration (reviewed in references 132 and 133). The concepts underlying this mechanosensing mechanism could be applicable in fungi if the cell-derived secreted proteins of the cell wall are considered as analogous to the mammalian ECM. Another concept associated with integrins is their linkage to the cytoskeleton, where contact is signaled via deformation of the cytoskeleton. Some protein components of the focal complexes through which mammalian integrins function are conserved in fungi, although their function has not been elucidated. Paxillin is the central scaffold protein and orthologs have been identified in *S. pombe*, *S. cerevisiae*, and *Ashbya gossypii*, where they generally localize to sites of new cell wall deposition such as cell tips and septa (134–137). However, orthologs of vinculin and talin, the components that link integrins to the cytoskeleton in the mammalian complex, appear to be absent. Importantly, proteins with integrin-domain homology have not been identified in fungi, despite various attempts to find them. Experiments where anti-(human) integrin antibodies bound to fungal cells were ultimately unable to pinpoint candidate proteins (138). Integrins generally bind to RGD moieties in ECM proteins, and synthetic peptides containing RGD sequences were able to inhibit the formation of appressoria in *Uromyces*, suggesting that a potential signaling pathway through integrin clustering was blocked (139). Affinity purification using two different β-integrin antibodies isolated a 95-kDa protein, but this has not yet been identified. In *Candida* spp., antibodies against human αM, αX, α5β1, and αVβ3 were found to interact with moieties on the fungal cell surface (138, 140–142). However, these were not identified and later evidence suggested that the binding proteins may be wall-associated GAPDH and alcohol dehydrogenase

(143, 144). A cDNA probe encoding a region from the integrin transmembrane domain, but not the defining extracellular head groups, of human αM identified a *C. albicans* protein, Int1, as a single-pass transmembrane protein containing several motifs common to αM (145). CaInt1 localized to the cell wall where it appeared to be involved with adhesion, because its deletion reduced binding of *C. albicans* to epithelial cells by 39% (146). Int1 was also involved in morphogenesis in some media and colocalized with the intracellular septin, Cdc3 (147). Because of the thickness of the fungal cell wall, at 50 to 100 nm (in *C. albicans*), it seems unlikely that a protein with extracellular binding domains could signal contact with a ligand through the cell wall matrix. An alternative possibility is that Int1 is bifunctional and, like many cell wall proteins, can be exported and processed by the still-unknown cleavage mechanism to become a wall-linked adhesin. To date, its role in contact sensing has not been examined. If functional homologs of integrin-like proteins operate as mechanosensors in fungi, they do not seem to be identifiable through protein sequence homology. Nevertheless, proteins with a homologous function may have evolved separately in fungi to act as a linkage system between the cell wall and the cytoskeleton.

FUTURE PROSPECTS

Evidence of the importance of the fungal sense of touch abounds in studies of fungal growth and behavior. While we can observe the outcomes following contact events, no definitive contact sensor has been identified. Fungi are not the only system in which this subject is under scrutiny, and, in some ways, they are better placed than bacteria or plant cells for advancing our understanding because of the combined advantages of genetic methods and the ability to correlate cell behavior with outputs from reporter proteins using live-cell imaging. One of the immediate challenges is the current lack of a comprehensive model of cell wall dynamics, its spatial organization at the molecular level, and the regulation of its viscoelastic properties. In addition, the function of many of the proteins isolated in cell wall proteomes remains uncharacterized. A better understanding of these areas might enable us to assess the likelihood that contact is sensed through a cell wall-embedded mechanism. Studies of the effect of carbon source on the cell wall during osmotic stress, where the cell size reduces by 50% in approximately a minute without buckling, and live-cell imaging of thigmotropic growth, have forced us to move away from the "rigid" cell wall model to one whereby cell wall organization renders it capable of rapid change (7, 70). This might support contact-induced local remodeling that is somehow transmitted to spatially organized intracellular proteins to define a zone of contact.

Ion flux, particularly calcium transients, are well recognized as mediators of mechanosensing in mammalian cells and bacteria. The use of genetically encoded ion reporters is set to revolutionize our understanding of calcium flux in fungi, which has been previously hampered by the failure of fungi to retain calcium dyes in the cytoplasm of living cells. With the advent of a range of new molecular, genetic, and imaging tools, an understanding of how contact is sensed by fungi is at last within our grasp.

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Melanin, Radiation, and Energy Transduction in Fungi

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INTRODUCTION

Melanins are dark pigments that are made by diverse fungi (1, 2). Even fungi that produce white colonies, such as *Candida albicans*, have the ability to make melanins (3, 4). Melanins have elicited considerable interest in microbial pathogenesis because they are important virulence factors for many pathogenic microbes, and their presence is associated with reduced susceptibility to antifungal drugs (5, 6). Melanins are multifunctional molecules that give cells structural strength as well as reduced susceptibility to temperature extremes, heavy metals, and molecules produced by the immune system such as oxygen- and nitrogen-derived oxidants and microbicidal proteins (2, 7–10).

Despite the importance of melanins in biology, their structure remains largely unsolved (11). Melanins are composed of covalently polymerized indole- and phenol-type compounds resulting in a material that is insoluble and acid resistant. Melanin structures exhibit local order with global heterogeneity, thus resulting in an amorphous material. Insolubility combined with an amorphous nature means that the structure of melanin cannot be solved with currently available analytical techniques such as X-ray diffraction. A remarkable property of melanins is that they are stable free radicals and manifest a distinctive electron spin resonance signature that is used in their identification (12). Despite the difficulties involved in working with melanin, considerable progress has been made in eliciting its structure by combining results from several techniques including solid-state nuclear magnetic resonance (11, 13, 14).

Melanin production in fungi is a result of three pathways known as the polyketide, 3,4-dihydroxyphenylalanine (L-DOPA), and L-tyrosine degradation synthetic pathways, which produce two chemically different compounds with similar properties (2, 7, 15, 16). Melanin synthesis involves free radical reactions with toxic intermediates. Consequently, melanin synthesis in both animals and fungi occurs in specialized structures known as melanosomes (17). Melanin can be located internally or in and on the cell wall, where it is closely associated with other cellular components such as lipids, carbohydrates, and proteins, although the nature of these associations is poorly understood due to the inherent difficulties in studying melanin structure. For *Cryptococcus neoformans*, one of the fungi in which the process of melanization is best understood, melanin synthesis is catalyzed by a laccase, and the pigment is exclusively 3,4-dihydroxyphenylalanine-melanin. *C. neoformans* melanin is synthesized in vesicles that are exported to the extracellular space and are assembled in the cell membrane into concentric layers, where the pigment is closely associated with polysaccharide and other cellular structures such as chitin and its derivatives (11–14).

Among the remarkable properties of melanin is its capacity for energy transduction (18). Melanin is unique among biological compounds in that its absorption spectrum reveals absorption of all wavelengths in the UV-visible-infrared spectrum. The capacity of melanin to absorb all these wavelengths is presumably a function of its complex molecular structure, which allows it to interact with these frequencies of light. The

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ability of melanin to absorb electromagnetic radiation extends into the range of X rays and gamma rays, such that it has a shielding capacity that is approximately half that of lead and twice that of carbon (19). Mice given fungal melanin are capable of surviving lethal doses of gamma irradiation, presumably as a result of the pigment protecting the digestive tract and associated lymphatic tissue (20). The ability of melanin to absorb and convert electromagnetic energy is associated with a variety of biological effects that give melanotic fungi tremendous resilience, which translates into their ability to survive in hostile environments.

MELANOTIC FUNGI INHABIT RADIATION-EXTREME ENVIRONMENTS

Melanotic fungi inhabit some of the most extreme environments known, and it is generally believed that the presence of melanin contributes to survival through a variety of mechanisms. For example, Antarctic black rock fungi include a large number of diverse melanotic species that colonize the rocks of one of the most extreme environments on Earth (21). For the purposes of this article we review the literature reporting melanotic fungi in high-radiation environments. The remarkable finding that the damaged reactor at Chernobyl and surrounding soils host a large population of melanotic fungal species is perhaps the best-known example of fungi living in a high-radiation environment (22–24). The Antarctic highlands, where many black rock fungi thrive exposed on the surface and interior of rocks, is also a high-radiation environment (21), especially during the long austral summer and considering the recent historical weakening of the atmospheric protection in the southern hemisphere through ozone depletion. Notably, black fungi recovered from Antarctica can survive in simulated Mars conditions (25). The melanotic fungus *Ulocladium chartarum* was able to grow under space flight conditions, with a rate of growth that exceeded that achieved on Earth (26).

MELANOTIC FUNGI RESPONSES TO RADIATION

Three lines of evidence indicate that melanotic fungi respond to radiation: kinetic attraction toward radioactive sources, faster growth, and metabolic changes. Although these lines of evidence are not completely independent (e.g., hyphal growth toward a radioactive source and enhanced colony growth both reflect cell growth, which in turn is associated with metabolic changes), they are sufficiently distinct to discuss separately.

Radiotropism

The term "radiotropism" refers to the ability of some fungi to grow toward radiation sources (27, 28). This phenomenon was first reported among fungal species colonizing the damaged reactor at Chernobyl and involved the migration toward and degradation of carbon containing "hot particles" (28). Radiotropism was triggered primarily by gamma radiation, but it is possible that both alpha and beta radiation could be "sensed" by the fungi as well. Although the mechanism and purpose of fungal cell migration toward a radioactive source remain unknown, the careful work documenting this phenomenon indicates that fungi respond to radiation and suggest intentional migration and attraction to this energy source.

Enhanced Growth

In 2007 our group reported differential growth effects on melanotic and nonmelanotic strains of *C. neoformans* and *Exophiala* (*Wangiella*) *dermatitidis* exposed to gamma radiation such that the melanin-containing strains grew faster than albino strains (29). This phenomenon was subsequently confirmed and expanded (see below) by another group (30). Enhanced fungal growth in melanotic fungi in response to high-energy electromagnetic radiation has also been reported for other fungal species. Large increases in colony-forming units (>100-fold) were observed for *Alternaria alternata* exposed to gamma radiation (31). Exposure of *Aspergillus versicolor* to radioactive nuclides increased hyphal length and spore germination, phenomena that were attributed to enhanced growth by radiation (24). Hence, enhanced growth of melanotic fungi in high-radiation environments has been observed for several fungal species by at least four independent groups. Analysis of the energy levels that triggered radiation-related fungal growth revealed that whereas low levels of radiation (150 peak kilovoltage [kVp]) enhanced the growth of both melanized and nonmelanized *C. neoformans*, higher levels (320 kVp) triggered enhanced growth only in the melanized cells (32).

Metabolic Changes

Analysis of wild-type and albino mutant *wdpks1 E. dermatitidis* strains' gene expression after exposure to high-energy radiation revealed differential expression of about 3,000 genes involving multiple pathways, such that genes for cell cycles of amino acid synthesis were downregulated, while stress response genes were upregulated (30). Of particular interest was the observation that the wild-type (melanotic) but not the albino *wdpks1* strain manifested ribosomal biogenesis genes'

upregulation to radiation exposure, leading the authors to suggest the possibility that melanin-derived energy was being used for protein synthesis (30). An experiment with three melanotic fungal species in a simulated Mars environment that included exposure to 200 nm UV radiation revealed some initial changes in protein expression followed by adaptation with resumption of normal expression, leading the investigators to conclude that these organisms could survive in that environment (33). An analysis of the response of *Hormoconis resinae* to chronic radiation resulting in cumulative doses of 2 to 3 Gy found that radiation was associated with enhanced synthesis of melanin and antioxidant enzymes (34).

INTERACTIONS OF MELANIN WITH HIGH-ENERGY ELECTROMAGNETIC RADIATION

For melanin to capture electromagnetic radiation in a manner that is suitable for conversion to biological energy, it must interact with it. Whereas melanin is known to absorb all UV, visible, and infrared frequencies, the phenomena of radiotropism and enhanced growth upon exposure to gamma rays depend on the ability of the pigment to interact with much higher-energy radiation. Such interactions have now been demonstrated by several methodologies. Irradiation of melanin with gamma rays resulted in changed electronic properties as measured by electron spin resonance spectra (20). A demonstration of the capacity of melanin to serve as an energy transduction molecule for high-energy electromagnetic radiation came from the observation that an electric current was produced by a melanin electrode placed in a gamma-ray beam (35).

IMPLICATIONS OF MELANIN-MEDIATED ENERGY TRANSDUCTION

The phenomenon of radiation-induced growth in melanotic fungi was called radiosynthesis in analogy to photosynthesis, by which plants convert light energy into chemical energy that they can utilize for biological processes (29). Supporting this designation was the observation that irradiated melanin was able to reduce NAD to NADH, thus providing a critical link for the conversion of electromagnetic energy into chemical energy that was immediately biologically useful. However, the analogy of radiosynthesis to photosynthesis is only partial, for the latter is understood to include a series of complex reactions that involve the fixing of carbon to synthesize new biologically useful molecules. For fungi, there are some suggestions that they can utilize CO_2 for

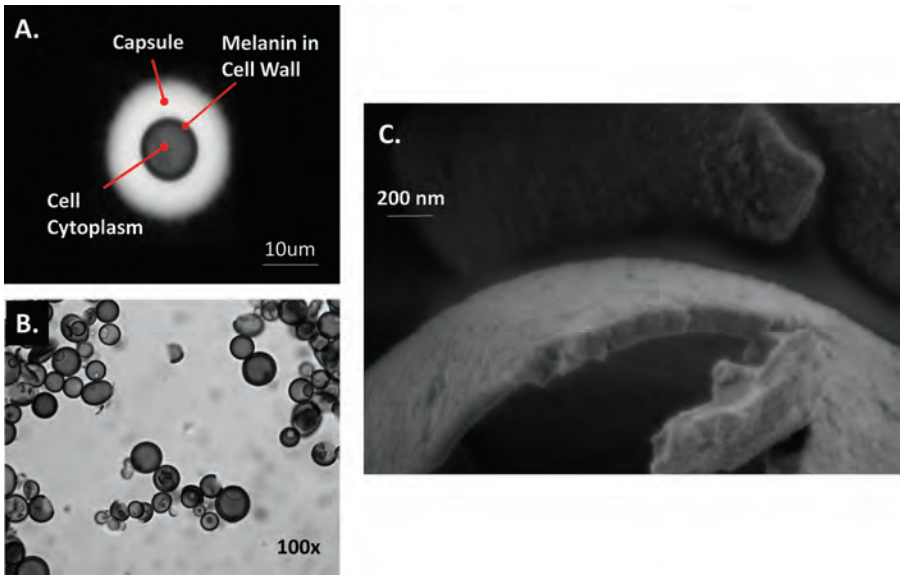


Figure 1 Melanized *Cryptococcus neoformans* and melanin "ghosts." (**A**) Melanized *C. neoformans* cell in India Ink suspension to illustrate the cellular location of melanin. The cell wall appears dark because of the presence of melanin. Note that melanin is external to the body of the cell. (**B**) Melanin ghosts prepared from melanized cryptococcal cells by strong acid digestion (38), which removes cellular components except for the cell wall-associated melanin. (**C**) Scanning electron microscopy of a melanin ghost showing its granulated surface structure, porosity, and thickness.

synthesizing organic molecules, but this topic has not been thoroughly explored. Studies of black rock fungi from Antarctica using ^{14}C suggested that they could directly utilize CO_2 and incorporate carbon into organic molecules (36). Earlier studies of the mold *Zygorrhynchus moelleri* provided evidence for the direct nonphotosynthetic incorporation of CO_2 into pyruvate (37). Hence, one can imagine a situation in an extreme environment such as Antarctic mountains where the combination of radiation harvesting by melanin combined with some capacity for utilizing inorganic carbon could produce a process akin to radiosynthesis.

MAJOR QUESTIONS AND FUTURE RESEARCH DIRECTIONS

At this time there is experimental evidence for the following observations: (i) melanin is an energy-transducing molecule with the capacity to absorb a broad spectrum of electromagnetic radiation; (ii) melanotic fungi exposed to high-energy radiation grow faster than exposed nonmelanotic mutants and unexposed fungi, both melanotic and amelanotic; (iii) melanin can interact with high-energy electromagnetic radiation and transduce it to chemical and electrical energy. In aggregate, these observations suggest that melanotic fungi have the capacity to utilize high-energy electromagnetic radiation to sustain some biological processes. This, combined with the possibility that fungi have some capacity to fix carbon, raises the possibility of radiosynthesis. However, we lack information about the detailed processes by which electromagnetic energy is harvested and converted into biologically useful energy. The ability of irradiated melanin to convert NAD to NADH *in vitro* suggests that it may contribute to energy utilization through simple oxidation-reduction reactions that harvest electrons for biological use. However, there is also a possibility that melanin-related energy utilization involves a sophisticated molecular apparatus such as an antennae complex to shuttle electrons from extracellular cell wall-associated melanin to the cell interior.

The biological advantages of melanin-mediated energy transduction are readily apparent. For organisms in extreme environments such as black rock fungi of Antarctica, the ability to harvest some light energy for biological processes could provide a tremendous survival advantage relative to nonmelanotic organisms. However, the exact contribution of this mechanism relative to the energy available from conventional nutritional processes available to fungi from other sources such as degradation of plant matter is unknown. Establishing the importance of this mechanism in fungi is a much more difficult undertaking than showing the importance of photosynthesis in plants, because the latter are totally dependent on sunlight for their survival, whereas fungi can access nutrients from other sources. In this regard, it is noteworthy that experiments showing that electromagnetic radiation enhances growth have relied on measuring growth increments relative to nonirradiated conditions or albino mutant controls rather than establishing an absolute requirement for growth, since the latter criterion has been experimentally difficult. Consequently, establishing the importance of melanin energy capture is an open question that will probably require innovative experimental design.

IMPLICATIONS FOR BIOLOGY AND EXOBIOLOGY

Given the widespread abundance of melanotic fungi in the biosphere, any amount of conversion of electromagnetic energy into biologically useful energy is likely to have a major impact on estimates of planetary energy flows. For example, photosynthesis is estimated to convert approximately 130 terawatts of sunlight into biologically useful energy, an amount that could increase significantly with melanin-related energy conversion. The ability of melanotic fungi to harvest energy would make them autotrophs and place them alongside plants as important contributors to the conversion of solar and physical electromagnetic energy sources into biologically useful energy. Because the energy of the ionizing radiation is several orders of magnitude higher than the energy of white light, even a very inefficient mechanism of its harvesting will still produce energy transfer. The fact that melanin pigments are easy to synthesize and are found in all biological kingdoms raises the tantalizing possibility that melanin-related energy transduction is ancient, predating photosynthesis, and served as a significant energy-harvesting mechanism for early life on Earth, which emerged in conditions of much higher radiation exposure.

Melanin-related energy transduction has immediate implications for exobiology since it implies that this pigment not only confers the capacity for survival in extreme environments but also provides a means for harvesting electromagnetic energy. The survival of melanotic fungi in simulated Mars-like conditions suggests that the resiliency conferred by melanin already provides some Earth microbes with the capacity to survive on other worlds, thus extending the limits of terrestrial life.

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Making Time: Conservation of Biological Clocks from Fungi to Animals

24

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INTRODUCTION

The Biology of Circadian Rhythms

What are circadian rhythms?

Despite years of study and a great deal of exposure in the scientific press, there is still sometimes confusion regarding what are the defining characteristics of a rhythmic process that makes it truly circadian. The essence of this is that the rhythm must be shown not to depend in any way on continued environmental cycles, and to have a period of about a day. So, in this way, it is obvious that in petri dish cultures grown under a light-dark cycle, a rhythmic change in orange color in *Neurospora crassa*, or in melanin in *Aspergillus fumigatus*, could not be categorized as circadian, because the change in pigmentation would be seen as the result of light induction of, respectively, carotenoid or polyketide synthesis. As humans, our pupils constrict in proportion to the amount of light to which the eye is exposed, so a daily rhythm in pupil size would not be circadian but would instead reflect a diurnal cycle in ambient light. This part of the definition identifies the rhythm as truly endogenous in nature, in this way being the overt expression of an internal circadian oscillator. Beyond this, the definition is more subtle and reflects the biology of rhythms.

What does it benefit a cell to have a circadian oscillator and not simply an hourglass timer that is set at environmental transitions such as dusk or dawn? There are at least two answers. One is that an oscillator does not need to be restarted every day; it will continue even without a time cue, for instance, even if the organism (like a fungus) is growing underground. Another answer is that it provides an additional level of flexibility in responding to a changing environment. In this context, we should briefly talk about phase and phase angle (Fig. 1). Every rhythm perforce has a peak and a trough as well as other defining points (e.g., point of sharpest decline, midway to peak, etc.), and, in general, such definable points are called phase reference points. In circadian biology, phase or phase angle is defined as the difference in time between the phase reference points of the environmental cycle and the circadian cycle. For instance, in *Neurospora* growing on an agar surface or a piece of bread in a light cycle consisting of 12 h light and 12 h dark, the number of aerial hyphae and conidia peaks about 2 h before dawn, so, if the phase reference point of the environmental cycle is dawn and the phase reference point of the circadian cycle is the peak of conidiation, then the phase of conidiation is about −2 h (i.e., before dawn), which is the same as +22 h (after dawn) on a 24-h cycle. When free-running under constant conditions, two rhythms can have the same period length but have different phases, because the peaks occur at different times with respect to the last light-to-dark transition. For a rhythm to be useful to the organism, it must be able to control activities so that they happen at appropriate times of day, and appropriate means not just a given number of hours after dawn or dusk but, more often, for example, the middle of the day period or night period, a point that can be a different number of hours after dawn or dusk depending on the season. To achieve this, circadian rhythms are entrained to environmental cycles as distinct from synchronized with them. Synchronized

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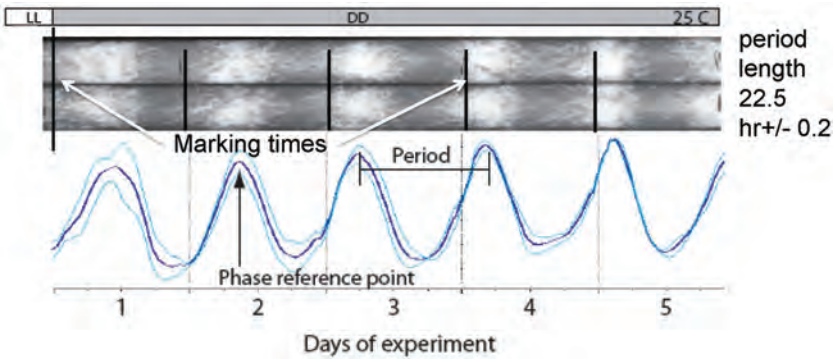


Figure 1 *Neurospora* circadian output as seen by control of growth and development in a culture growing across an agar surface. Cultures are inoculated at the left and grow to the right. After a period in constant light, the culture is transferred to constant darkness. The position of the growth front at the time of transfer is marked and is marked again every 24 h, giving a measure of time since the light-to-dark transfer. Below the images of the cultures is a densitometric scan of the mycelial/conidial optical density. The peak of conidiation happens roughly 10 h after the light-to-dark transfer and recurs after that roughly every 22.5 h (one circadian day) for cultures grown at 25°C. Typically, the peak in conidiation is used as a phase reference point, but any definable point in the cycle can be used. Adapted with permission from reference 49.

would mean that the rhythm tracks the external cycle and assumes a fixed phase and period length of the external cycle, whereas entrained means that the rhythm assumes the period, but the phase reflects interplay between the circadian oscillator and the environmental cycle. The value of this is that the circadian system can evolve (and has) to preserve phase across the spectrum of light-dark cycles seen in a year. At the temperate latitude in Vermont where we live, daylight can range from 8 to 16 h depending on the season. The circadian system can keep an important biological activity (say conidiation in *Neurospora* or sleep in people) occurring at roughly the same point in the cycle, such as late at night, despite changes in the light-dark cycle. As described in more detail later in the review, in *Neurospora*, a large variety of processes ranging from gross characteristics, such as sexual and asexual developmental potential, growth rate, and the cell cycle, to gene expression and fluxes through metabolic pathways, are all coordinately regulated by the circadian system. This coordination is adaptive because it helps the organism to organize its growth and metabolism in anticipation of dependable environmental changes such as temperature, ambient light, and UV stress.

Circadian homeostasis

Another environmental variable that has profound impact on biological systems is temperature. For a clock to be useful in the context described above, namely, being sure to keep important processes happening at the right time(s) of day, it would not do for the rate of progression of the clock to be strongly influenced by ambient temperature. Physiologists and biochemists often frame general discussions of temperature effects on rates of processes in terms of Q_{10}, the rate of a process at one temperature divided by the rate at 10°C lower temperature. A typical enzymatic reaction will have a Q_{10} of 2, and a complex set of reactions such as mitochondrial respiration may have a Q_{10} of 4. Imagine the effect on phase angle if the clock ran three times faster during a 30°C day in July as on a 15°C night. Circadian rhythms are special because they have evolved to have a Q_{10} of close to 1. A process with a Q_{10} of exactly 1 would be temperature independent; circadian period lengths are not temperature independent, and even in the same organism across some ranges, the Q_{10} can be slightly less than 1 and, across other ranges, slightly greater than 1. For this reason, circadian period lengths are said to be temperature compensated.

Another aspect of circadian homeostasis is that circadian period length is resilient to the effects of changing nutrition. This again makes sense ecologically, especially for a fungus that can encounter enormously variable nutritional conditions over the course of weeks as it moves from fresh to rotting to desiccated substrates. Temperature and nutritional compensation distinguishes circadian rhythms from other ca. 24-h rhythms such as the mammalian cell cycle.

Time cues for keeping internal time in sync with external time

While circadian period length needs to be resilient to the effects of ambient temperature, it is immediately plain that the clock needs to be sensitive to daily changes in a variety of environmental variables so that internal time can be a proxy for external time. In most organisms, daily changes in light intensity set the phase of the internal clock, with lights-on corresponding to dawn and lights-off corresponding to dusk. All circadian organisms including fungi have evolved ways to detect even low levels of light and to use changes in intensity to set their clocks. Indeed, humans and fungi use light in the same way, and the understanding of how light is used to reset the circadian clock was first elucidated in *Neurospora* as discussed later. Clocks are sensitive to temperature changes to the extent that they can also be used as time cues for day and night. Indeed, in many organisms (including *Neurospora*), temperature can be a stronger time cue than light.

The Definition of Circadian Rhythm

To paraphrase, we have defined a true circadian rhythm in this way: a circadian rhythm is an oscillation in a biochemical, physiological, or behavioral function that persists under conditions of constant light or darkness, temperature, and other environmental variables with a period length of about a day, whose phase can be reset by a brief interruption in the constant regimen, and whose period length is relatively independent of temperature and nutrition within the physiological range of normal growth (1). Thus, these rhythms are not a simple response to the environment but are phased by it. Overall, it is these few characteristics—period length of about a day, persistence under constant conditions, resettable by changes in this constant regimen, and compensated against ambient changes in temperature or nutrition—that define a rhythm as being circadian. Rhythms having these characteristics, whether in fungi or animals, are united by a common biology and, as it turns out, by a common molecular mechanism. These are also the characteristics that molecular models for the circadian oscillators have sought to explain. Specifically then, there have been three salient questions: How does the oscillator work (mechanism)? How do environmental cues impart time-of-day information on the oscillator (input)? How does the oscillator control the behavior and metabolic potential of the cell (output)? Work on *Neurospora* played *a*, or in some cases *the*, leading role in answering the first two of these questions and remains at the forefront of answering the third.

Circadian Rhythms in Fungi

Many fungi can express one (or even more than one) kind of rhythm—growth, morphology, or even in gene expression—if the medium and environmental conditions are just right (2–4). In the vast majority of cases, these are not truly circadian by the definition described above, and, in fact, true circadian rhythms in fungi have been described in only a few organisms including *Neurospora* spp. (4) and the closely related *Sordaria fimicola* (5), *Aspergillus flavus* (6), *Cercospora kikuchii* (7), *Botrytis cinerea* (8, 9), *Pyronema confluens* (10), and the basidiomycete *Neonothopanus gardneri* (11) where the circadian clock controls fungal bioluminescence and is thought to help with spore dispersal. Extensive effort has failed to reveal convincingly a rhythm in *Aspergillus nidulans* or *A. fumigatus* (although a rhythm in *gapdh* mRNA has been reported in *A. flavus* [6]), and reports of circadian rhythms in the yeasts *Schizosaccharomyces* (12) and *Saccharomyces* (13) have been questioned or not been reproducible. Given the extensive conservation of the core clock elements FRQ, WC-1, and WC-2 within the Ascomycota, in general, and the Sordariaceae, in particular, it seems likely that rhythms could be identified in many additional organisms. A wonderful example of this is the recent description of a canonical FRQ/WCC-based clock in the pathogen *B. cinerea* that influences its pathogenesis (8, 9).

The field of rhythms in fungi other than *Neurospora* has been excellently reviewed elsewhere recently, and interested readers may want additional perspectives (8, 14–20). While rhythms can include both circadian and infradian noncircadian cycles, more attention will be paid to the relatively extensive literature describing noncircadian rhythms in fungi near the end of this review. Before that, the focus will be on the true circadian rhythm in *Neurospora* and what it has shown about circadian rhythms in general.

THE *NEUROSPORA* CIRCADIAN OSCILLATOR

The historical development of this field has been quite interesting but has been adequately reviewed elsewhere (21–29) and will not be redeveloped in depth here. Instead, we will aim for a succinct and comprehensible (at the expense of comprehensive) overview of how the circadian system works.

WCC Drives *frq*

The white collar complex or WCC comprises WC-1 and WC-2. WC-1 is a GATA-type transcription factor

having a Zn finger DNA binding domain and three PAS-like domains, amino acid sequences often associated with protein-protein interaction or with ligand binding (30). One of these is a specialized PAS domain called a LOV domain, a subtype associated with light, oxygen, and voltage regulation. As discussed below under "Input," WC-1 is the founding member of the principal class of fungal blue light photoreceptors, an activity mediated by the LOV domain, although, for the circadian oscillator itself, the LOV domain is dispensable (31). The other PAS domains, however, are essential in that they mediate the interaction between WC-1 and its partner protein in the WCC, WC-2. Figure 2A shows a cartoon of the domains of WC-1 and WC-2 and describes the activities associated with these domains as well.

The WCC binds to two places in the promoter of the *frequency* (*frq*) gene (32) (Fig. 2B). One is the proximal light regulatory element (PLRE) that is very near to the transcription start site (TSS) and that is required for the acute induction of *frq* expression by light that forms the basis of light resetting of the clock (see "Input," below). Binding to another element more than a kilobase distal (5′) from the TSS, the clock box (C-box) is required for circadian activation of *frq* expression as a part of the circadian oscillator. Binding of WCC to the C-box displaces local nucleosomes (33) and requires the Zn fingers of both WC-1 and WC-2 as well as a basic region of WC-1 just N-terminal from the WC-1 Zn finger, a region called the DBD because proteins lacking it are defective in DNA binding (34). One might reasonably ask how binding of WCC to the C-box can effect transcriptional activation if the site is more than a thousand bases away from the TSS. The answer is that an N-terminal region of WC-1 serves to attract the SWI-SNF chromatin remodeling complex (31), a group of proteins known to be involved in nucleosome removal and chromatin remodeling as well as in bending of DNA (35). Thus, the model is that SWI-SNF promotes bending of the DNA in the *frq* promoter to bring the WCC bound to the C-box into proximity with the TSS where it can attract the general transcription factors and Mediator complex involved in initiation of transcription (31).

In addition to the transcription of *frq* in the sense (coding) direction, there is also a *frq* antisense transcript (36) named, appropriately, *qrf* (*frq* spelled backward) (Fig. 2B). The WCC binds downstream of the *frq* coding region where it serves to mediate light induction of this antisense transcript; the exact location of the

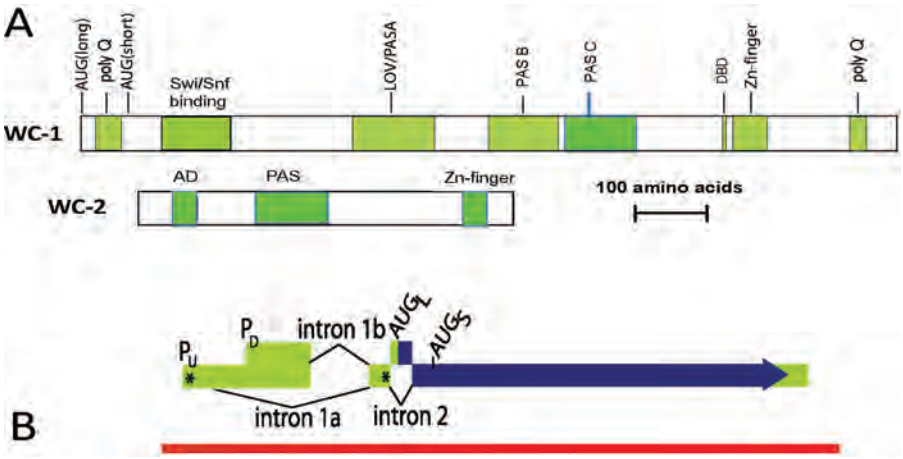


Figure 2 The WC-1 and WC-2 proteins and the *frq* locus. (**A**) Cartoon schematics of WC-1 and WC-2 are shown to scale. Landmarks include protein start sites (AUG) polyglutamine stretches (polyQ), the binding site for SWI/SNF (31), the LOV domain that mediates photoreception, the two PAS domains of WC-1 that mediate interaction with the PAS domain on WC-2, and the DBD domain that assists the Zn finger in DNA binding to the C-box. The putative transcriptional activation domain (AD) of WC-2 is marked. (**B**) Complex regulation and splicing involved in *frq* expression. The C-box and PLRE are upstream from P_U and P_D, the upstream and downstream start sites for transcription, respectively. The parts of the primary transcript left after splicing of intron are marked in green, asterisks mark upstream ORFs that influence the amount of FRQ made, and purple marks the FRQ coding region. Temperature-regulated splicing of intron 2 governs whether AUG_L or AUG_S is used to initiate FRQ. The *frq* antisense transcript, *qrf*, is marked in red. Adapted from reference 128.

promoter driving constitutive *qrf* expression has not been mapped. Xue et al. (37) have recently provided a nice rationale for the expression of the antisense *qrf* transcript: even in the absence of WCC binding to the C-box, there is a low level of sense strand *frq* expression, and the resulting low level of FRQ expression causes the oscillator to dampen. Constant low-level *qrf* expression runs counter to the sense strand expression and serves to reduce this background to near zero, thereby keeping the oscillator from damping (37). *qrf* expression also provides antisense transcripts that may elicit dicer-independent siRNA (disiRNA)-mediated facultative heterochromatin formation at *frq* and thereby aid in shutting down *frq* expression (38). WCC binding within the *qrf* promoter promotes light induction of *qrf*, and the spike of antisense transcript coincident with the spike of light-induced sense *frq* transcript aids in light resetting of the clock by making sure that FRQ appears as a pulse and not a tonic increase. Light resetting is compromised in the absence of *qrf* expression (36).

FRQ Is Made and Acts

frq mRNA moves to the cytoplasm for translation although there is a surprisingly long lag between when it first appears and when FRQ protein finally turns up several hours later (Fig. 3). The cause of this long lag is unknown, but it is likely a result of explicit regulation because, after acute light induction of *frq*, the FRQ protein appears well within an hour (39). The translation of FRQ has received some attention recently with interesting results. It has been known for a long time that the codon bias of *frq* is strongly skewed toward rarely used codons, those that require low-abundance tRNAs. Zhou et al. examined the importance of this by changing the codon bias in favor of those codons ser-

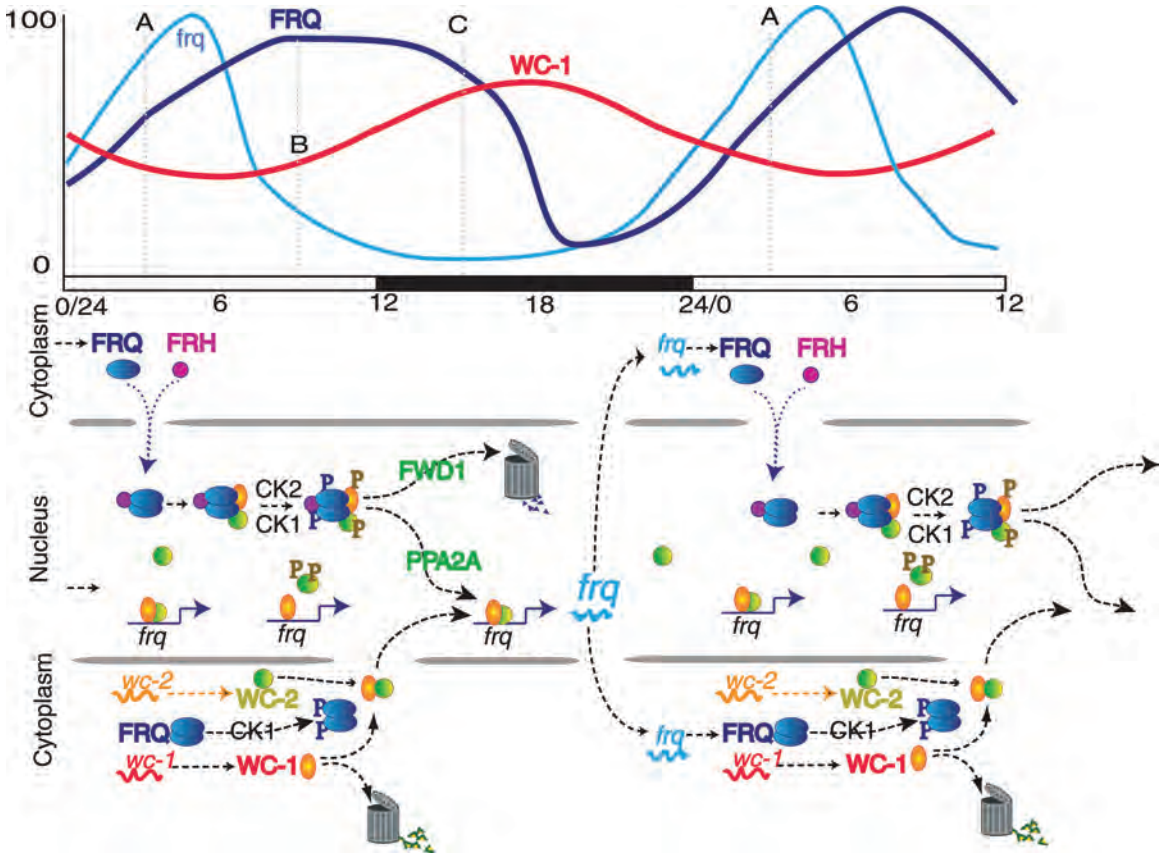


Figure 3 Temporal outline of the *Neurospora* circadian clock. (**Top**) Amounts of clock-relevant RNAs and proteins cycle. (**Bottom**) Shown as a function of time are the locations of RNAs and proteins important for the circadian oscillator. Time goes from left to right, beginning at subjective dawn (CT 0, about 11 h after a light-to-dark transfer). Trash can, proteasome; PP, phosphatases. Adapted from reference 24.

viced by abundant tRNAs and the result was more FRQ, as expected, but less functional FRQ: the FRQ produced by the codon-bias-"improved" gene was less stable, less able to enter into productive complexes, and less able to support its role in the oscillator (40). The model is that FRQ folds cotranslationally, perhaps promoted by interaction with other protein(s) as described below, and that this cotranslational folding and interaction require some pausing of translation as helped by the codon choice in the wild-type gene (41).

Upon translation, FRQ dimerizes and forms a complex with another protein, FRH. Dimerization of FRQ is essential (42) as FRQ's immediate interaction with its partner protein FRH. FRQ is an intrinsically disordered protein (IDP), meaning that, unlike more typical proteins, it has no inherent structure. Most proteins fold up into specific structures and can be thought of as oil droplets where hydrophobic residues are pushed away from water and into the center of the protein, allowing hydrophilic amino acid residues to be on the outside. For proteins such as these, the structure of the protein plays an essential role in its function and also contributes to its stability. When such proteins are heated so that the structure is disrupted and the hydrophobic residues face outside, the protein becomes denatured and falls out of solution; think of the albumin of a cooked egg. IDPs, however, have no inherent structure, so, when heated, they remain in solution, but they are also subject to rapid proteolysis in the cell unless they are protected by another protein—enter FRH.

Two Cellular Roles for FRH

FRH, the FRQ interacting RNA helicase, was identified in 2004 as an essential protein that interacts stoichiometrically with FRQ (43). As its name implies, FRH has a "normal function" in a subcellular structure called the exosome that plays a role in the turnover of RNA (44), specifically in unwinding the RNA in a process driven by the hydrolysis of ATP. FRH belongs to a well-studied family of such ATP-dependent RNA helicases, several of which have crystal structures and all of which bear conserved sequences that are essential for their enzymatic functions including binding and hydrolyzing ATP. With all this evidence pointing to an enzymatic function for FRH, it was natural to assume that the role of FRH in the clock was enzymatic; surprisingly, however, this is not so. In a startling turn of events, mutants of FRH that have lost their required catalytic residues have been shown to still function normally in the clock (45). FRH is thus understood to play a purely structural role, stabilizing the IDP FRQ and protecting it from proteolytic turnover.

Processive and Choreographed Phosphorylation of FRQ

The observant reader will be asking how FRQ can do what it does if it has no structure and is just a random coil of amino acids in a chain floating in the cytoplasm. The answer is that FRQ is one of the most heavily post-translationally modified proteins known, and it is almost certainly these modifications that lead FRQ to participate in the diverse time-of-day-specific interactions that lead to a functional clock. A cartoon structure of the functional domains of FRQ is shown in Fig. 4. FRQ is phosphorylated at over 100 serine and threonine residues across much of the protein, but this happens very gradually over the course of the circadian day and in a highly choreographed fashion (Fig. 4), such that some regions of the protein become highly phosphorylated before other regions are significantly modified (46–48). It is very likely that the addition of the highly charged phosphate groups changes the local charge of the protein, and this suggests that each wave of phosphorylations elicits changes in the local structure of FRQ and in its ability to interact with other proteins. Consistent with this finding, the mutation of phosphorylation sites within FRQ can speed up the cycle or slow it down depending on where the sites are and when in the cycle they are phosphorylated (Fig. 4). Indeed, this is seen when one looks at the spectrum of proteins with which FRQ interacts (Fig. 3). With the exception of FRH, these change in a time-of-day-specific manner. Early in the day, FRQ interacts strongly with WC-1 and WC-2 and, as the day goes on, this interaction decreases until, by the end of the day, there is no strength to the interaction above background. This seems to fit well with the net action of FRQ in inhibiting the transcriptional activity of the WCC. As soon as FRQ appears, WCC activity plummets with the result that *frq* mRNA is elevated for only a relatively short period of time, whereas FRQ protein is elevated for most of the day.

A bit later in the day, the interaction of FRQ and FRH with casein kinase I (CK1) increases, and, after this, CK1 is also a part of the complex containing FRQ, FRH, WC-1, and WC-2. These are the strongest interactions, strong enough to survive purification of FRQ through multiple steps in preparation for the mass spectrometry in which the FRQ-interacting partners were identified and the sites of phosphorylation determined (47). There are, however, very likely a number of additional kinases that interact more transiently with FRQ, because these have been identified explicitly with various functions or activities (24, 49–51). For instance, in addition to the data showing physical interactions,

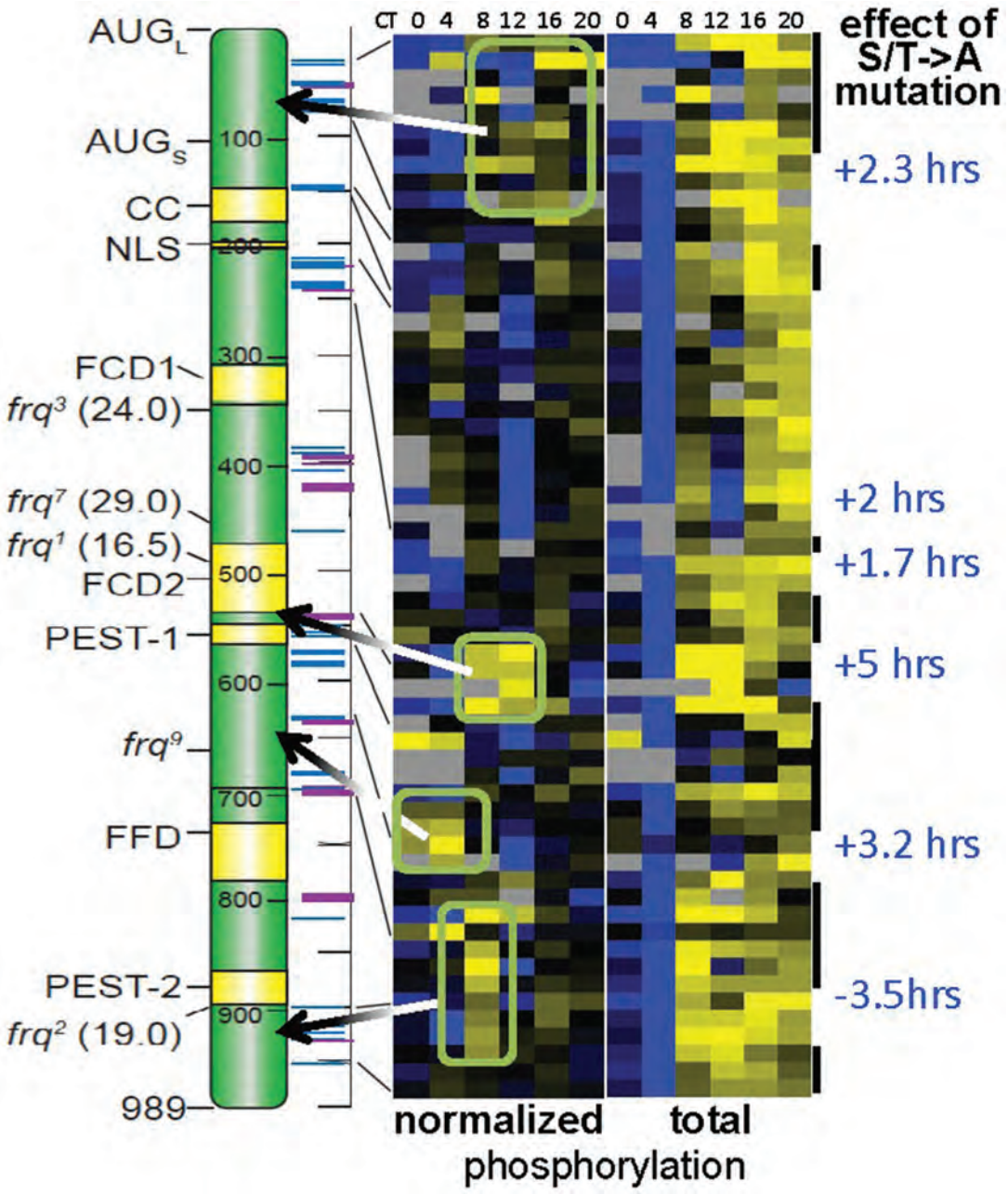


Figure 4 FRQ domains and their time-of-day-specific modifications. A cartoon of the domains of FRQ is shown to the left: CC, coiled-coiled domain; NLS, nuclear localization signal; FCD, FRQ-CKI interacting domain; FFD, FRQ-FRH interacting domain; PEST-#, PEST domains important for protein turnover; *frq*$^{\#}$, *frq* alleles followed by period length. Moving to the right in the figure, the positions of phosphorylations are marked. The two heat maps show how these change over time, with yellow marking higher phosphorylation and blue marking lower phosphorylation; each heat map consists of six columns corresponding to 4-h blocks of time in the circadian day from subjective dawn (CT0) through dusk (CT12) to the next night (CT20). The heat map to the right shows total phosphorylation, and, in the heat map to the left, the level of phosphorylation at any time is normalized to the total phosphorylation of the protein to better highlight where changes are occurring; these areas are marked with boxes and arrows showing where they lie within FRQ. Farthest to the right are noted the effects of mutating the serines or threonines that are phosphorylated at the locations noted to alanines. Modified from reference 47.

Neurospora strains bearing mutations in CK1 show abnormal period lengths (52). Likewise, casein kinase 2 (CK2) weakly interacts with FRQ and, importantly, CK2 has been shown to play a role in temperature compensation as described below (51, 53). PRD-4 in *Neurospora* is the ortholog of checkpoint kinase-2, a kinase activated by DNA damage. In *Neurospora*, the clock controls the cell cycle and DNA damage arrests the cell cycle in part through the action of CHK2, and also resets the circadian clock again through the action of CHK2 (i.e., PRD-4) (54). Protein kinase A (PKA) and calcium calmodulin kinase-1 (CAMK-1) have been implicated in FRQ phosphorylation and clock function, because mutants in them affect circadian period length and because they can phosphorylate FRQ *in vitro* (55). Relatedly, three protein phosphatases (proteins that remove phosphates from proteins) have been implicated in clock function (56). These are PP1, PP2A, and PP4; however, these may be indirect effects because *in vivo* analysis of FRQ phosphorylation revealed no strong evidence for dephosphorylation of any residues. Instead, the progression appeared to be unidirectional. Many of these protein-modifying enzymes have conserved roles in the circadian oscillators of animals as described later on.

FRQ Becomes Inactive, the Cycle Begins Again, and FRQ Is Turned Over

The function of the extensive posttranslational modifications of FRQ is plainly to regulate its activity, but a separate question concerns how the daily cycle is terminated. One of the first and most durable hallmarks of all circadian oscillators in the fungal and animal lineages is the observation of a daily cycle in the mRNA encoding the negative elements, accompanied by a daily cycle in their abundance (57–59). In addition in both *Neurospora* and *Drosophila*, there is an excellent correlation between the measured half-life of the negative elements (FRQ and PER, respectively) and circadian period such that longer half-life (more stable proteins) corresponds to longer period lengths (60, 61). This gave rise to the very reasonable assertion that the circadian cycle ends when the negative element is degraded, thereby releasing the positive element to begin the cycle of transcription anew. Despite the common restatement of this in nearly every clock review, recent work in *Neurospora* (62), while validating the correlation, has shown it to be just that, only a correlation, and that the underlying causal connection between clock protein stability and circadian period length does not exist.

This surprising turn of events began with the study of FRQ turnover that identified a ubiquitin ligase, FWD-1, as the enzyme responsible for ubiquitylating FRQ, the signal modification that targets it for protein turnover in the proteasome (63). In a very large number of proteins, FRQ included, phosphorylation of groups of amino acids in domains of the protein is the signal that targets a protein for ubiquitylation and turnover, so the extensive phosphorylation of FRQ fits this pattern well. A prediction, however, is plain, namely that mutations in FWD-1 that increase the half-life of FRQ must increase period length in a predictable manner, even yielding arrhythmicity, and, indeed, when examined on race tubes, strains lacking FWD-1 do not display a rhythm (63). However, surprisingly, when the clocks of such strains are examined using a luciferase reporter, an underlying rhythm is revealed (62). At the level of the oscillator itself, the rhythm is of normal amplitude, and, moreover, the canonical allelic series of short-period and long-period mutations in FRQ has the same effect on the oscillator, indicating that the clock in these strains is the same clock as is normally seen on race tubes. The feedback loop is being closed, determining period length, without a concomitant turnover of FRQ protein. How can this be?

The model proposed to explain these data (62) is that as FRQ (and, by extension, the other salient negative element clock proteins in animals such as PER in mammals) undergoes its unidirectional daily cycle in phosphorylation, its changes in charge allow it to participate in sequential structures and complexes that impact the activity of the WCC, and perhaps other enzymes. The cycle ends when FRQ becomes so phosphorylated that it no longer interacts with the WCC or other relevant proteins; indeed, by day's end, there is no longer any statistically significant interaction between FRQ and the WCC (47). Beyond this point, it does not matter if it is there, and, in fact, in the FWD-1 knockout strains, it is still there. Total cellular FRQ can have a very long half-life, but the clock as measured by luciferase runs along with little change, the small period differences being explained by effects of loss of FWD-1 on other substrates.

In wild-type strains, once FRQ has no further function and no further interactions with other relevant proteins, it is degraded; however, this act is viewed as only cellular housekeeping. By the time FRQ is being turned over, the WCC has already begun a new cycle of FRQ expression and a new circadian day has started.

Temperature and Metabolic Compensation

As described in "Circadian homeostasis" above, for a circadian clock to be truly useful to the organism, it needs to be compensated against changes in the ambient

environment. This is known as metabolic/nutritional and temperature compensation and is a core property of clocks as reflected by the fact that even isolated mammalian tissues and cells in culture display temperature compensation (64, 65). The mechanism for compensation is not known and, indeed, there are few clues, in part, because the experimental analysis is challenging. Rather than just determining the period length of mutant strains to see if compensation is altered, the period length must be determined by growing organisms at several different temperatures across the physiological range. Moreover, partial loss of compensation is commonly associated with period defects, suggesting that it may not be informative to the mechanism, if only because mutant proteins may have altered stability. In this case, a temperature gradient turns into a gradient in activity or effective dosage. However, in the study of compensation, *Neurospora* again led the way. Because loss of compensation mutations might not be informative, mutations yielding enhanced compensation were targeted for study. The *Neurospora* clock is normally modestly undercompensated, meaning that the period length becomes slightly shorter as the temperature increases from 20°C to 30°C. Two mutants, *prd-3* and *chr*, reverse this and instead show overcompensation or extended compensation (66). Cloning of these genes revealed them to encode separate subunits of casein kinase 2 (CK2): *chr* encodes the regulatory β subunit that also contributes to substrate recognition and *prd-3* is mutated within the catalytic core of the α subunit (50). The finding that the only two mutants known to cause overcompensation are subunits of the same enzyme is strong circumstantial evidence pointing to a role for CK2 in temperature compensation.

In many circadian systems, CK2 is known to play a circadian role. In *Arabidopsis* the β subunit CKB3 associates with CCA1 and overexpression of CKB3 leads to a shorter period (67); and, in *Drosophila*, CK2β and CK2α are defined, respectively, by *Andante* and *Timekeeper*, two long-period clock mutants. In *Neurospora*, FRQ is phosphorylated *in vitro* by CK2 and period lengthens with reduced dosage (53); although CK2α null mutants will not germinate, Δ*ckb-1* is viable but arrhythmic, the effect of CK2 dosage on compensation can be assessed in this background. When the regulatable *quinic acid-2* (*qa-2*) promoter (e.g., references 68 and 69) was used to drive *ckb-1* expression, leaky expression absent inducer causes a long 28- to 30-h period at 25°C (50). Interestingly, however, as CK2β1 levels are increased, the period drops to within the wild-type range, and strains expressing high levels of CK2β1 are slightly undercompensated like the wild-type clock; and, with less inducer as CK2β1 levels decrease, compensation passes through a range of better than normal compensation, finally changing to overcompensated mimicking the $ckb\text{-}1^{chr}$ overcompensation. Only the dose of WT proteins is being controlled in these experiments. The straightforward interpretation is that higher temperature results in more kinase activity (as confirmed in reference 50), and this acts to slow the pace of the clock at higher temperatures. These data establish a clear role for CK2 in effecting compensation.

INPUT TO THE CLOCK

Although much emphasis historically has been placed on the mechanism of the oscillator and the precision of the clock, it is the ability to be reset every day that may really be most important. Even a bad watch is still useful if it is corrected twice a day at dawn and dusk, and, in fact, this makes the clock useful in many different photoperiods spanning the seasonal differences from a winter of 6 h of light and 16 h of dark to the summer of long days and short nights. Clocks use available dependable environmental cues for clues as to time of day and these are known as zeitgebers from the German for time givers. The most dependable environmental cues are light and temperature, and these act on all known circadian clocks, although more complicated organisms such as people can also use derived cues such as feeding time or even social interactions.

Light

The mechanism through which fungal and mammalian circadian oscillators are reset by light was first elucidated in *Neurospora*. The key to understanding how this works is to understand the task in resetting. A single unidirectional environmental cue, the presence of light, must result in a bidirectional shift in the phase of the rhythm: if light is seen in the latter part of the night, it means that dawn has arrived and so the light signal should act to shift the phase of the clock forward to morning, whereas, if light is seen in the early part of the night, it means that the sun has not yet set, so the light signal should delay the clock back to the previous dusk. And of course, if light is perceived during the middle of the day it should have little effect on the clock.

This is achieved through the simple existence of a daily cycle in FRQ abundance and the fact that light induces the expression of *frq* mRNA and FRQ protein (Fig. 5) (70). Succinctly, in the daily cycle of *frq* mRNA abundance, it takes about 8 to 9 h to go from trough to peak and about 12 to 14 h to go from peak to trough.

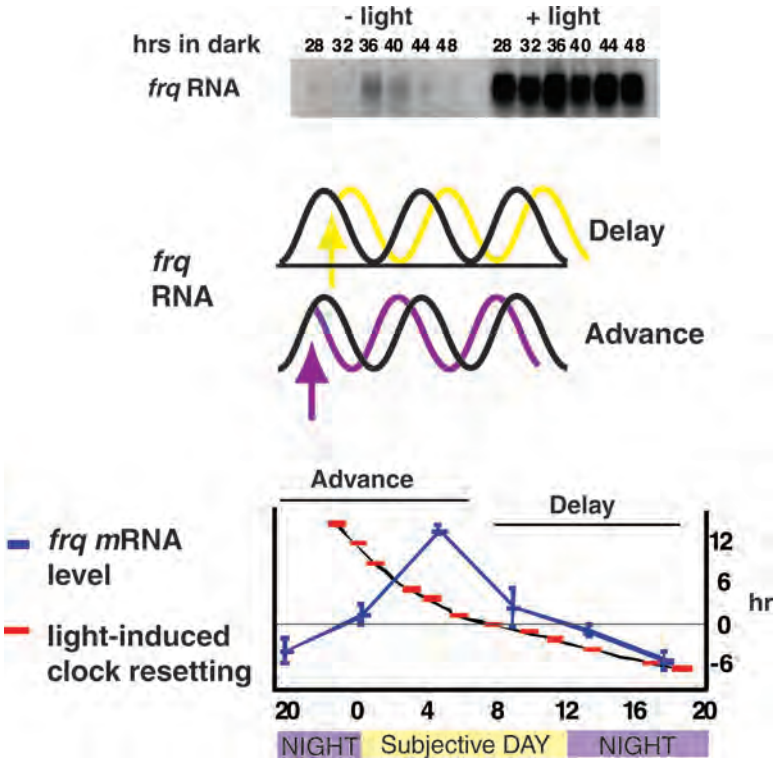


Figure 5 Light resets the clock. (**Top**) The daily cycle in the abundance of *frq* RNA is shown along with how a brief exposure to light at any time greatly increases this. (**Middle**) The cycle of *frq* transcription is shown in black, and the colored lines show the effect of light-induced *frq* expression on the steady-state rhythm. (**Bottom**) The response of the clock to light is juxtaposed with the daily cycle of *frq* expression: When light is seen while *frq* levels are rising, the phase of the cycle is advanced because *frq* rises to peak levels sooner. When light is seen while *frq* levels are falling, the cycle is set back. Adapted from reference 24.

If mRNA is rising slowly and the organism sees light, then *frq* is rapidly induced and rises; what would have taken hours now happens within minutes, essentially an immediate phase advance. Conversely, if *frq* mRNA levels are falling and the organism sees light, then *frq* is rapidly induced and the clock is set back to the time of peak. In constant conditions, peak *frq* mRNA levels happen in the subjective morning (the phase that would be morning on a light-dark cycle), so the effect of resetting is always to push the clock to subjective morning.

In *Neurospora*, the photoreceptor that brings about this acute light induction is WC-1 (32), the same transcription factor protein that heterodimerizes with WC-2 to make the positive element in the core feedback loop. There is a specific protein domain that senses light, the LOV domain, which is a specialized kind of PAS domain. The LOV domain binds a chromophore, FAD (flavin adenine dinucleotide) that strongly absorbs blue light (32, 71). When it absorbs blue light, it becomes chemically excited and makes a covalent adduct with a cysteine in the binding pocket of WC-1. By analogy to the VVD protein that also possesses a LOV domain and is a blue light photoreceptor (72), this event causes structural changes in the WC-1 protein that propagate to the surface of the protein where a helix is released, exposing an interaction surface. WC-1 interacts with itself (multimerizing [32]), exposing a transcriptional activation domain(s) that interacts with general transcription machinery to drive the acute induction of the *frq* gene, events that all happen within minutes of light exposure. These events happen at the PLRE in the *frq* promoter, a region that is close upon the transcription start site and, as described earlier, well separated from the C-box where WC-1/WC-2 (the WCC) mediate activation of *frq* as a part of the circadian cycle that happens in constant darkness.

Interestingly, clocks in mammals are reset in very much the same way, although they use a different means to detect light (73). As we all know, in mammals, photoreceptors for light are present only in the eyes, so

light perception elicits neuronal signals from each eye that travel down the optic tracks to the brain; the tracks cross at the optic chiasm. There in the hypothalamus, and directly above the optic chiasm, lie two bundles of neurons called the suprachiasmatic nuclei (SCN), about 10,000 each, that comprise the central pacemaker that controls locomotor activity and sleep-wake cycles. The light signal goes to a part of the SCN where it elicits release of the neurotransmitter glutamate, which elicits intracellular release of calcium ions, activating PKA and other kinases that phosphorylate and activate CREB (cAMP response element binding protein) and CRTC1 (CREB-regulated transcription coactivator 1), which bind to the *Per*1 and *Per*2 promoters and induce their expression, thus resetting the mammalian core clock (73, 74). Thus, although the means to achieve clock gene induction are different and reflect the biology of the cell types involved, the overall mechanism, acute light induction of negative elements in the core oscillator, is the same in *Neurospora* and in mammals.

Interestingly, the regulatory parallels between fungi and mammalian clocks do not end with the mechanism of light resetting; light also induces an acute repressor of the light response. This was also first elucidated in *Neurospora* where light acutely induces *vivid* that encodes VVD, another photoreceptor protein that acts as a negative regulator of the WCC (75). VVD physically interacts with WC-1 (76–78) and dampens its activity, and this has two effects. First, VVD turns what would be a step increase in WCC activity into a pulsatile increase followed by a return to background levels of activity. Second, VVD facilitates a graded response to successively higher light responses; this is because VVD binds to light-activated WC-1 and sequesters it, and the next higher intensity light signal then releases some of the sequestered WCC to allow reinduction of light-regulated genes (79). In mammals, light-driven CREB activity induces Sik1 (salt-inducible kinase1), which inactivates CREB through phosphorylation (80). Thus, as with acute light induction of circadian negative elements, there is conservation of the regulatory architecture of the light response in mammals to achieve the same end, turning what would be a step increase into a pulsatile increase.

Temperature

Temperature is the environmental variable, other than light, that is most dependable on planet Earth and, as a result, it influences phase of all known circadian clocks. Interestingly, and despite our well-described mechanisms for whole-body temperature homeostasis, this even extends to clocks in isolated human cells.

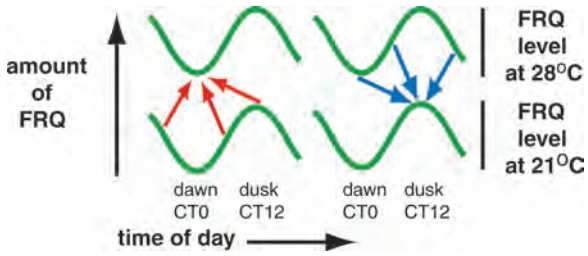


Figure 6 How temperature might reset the circadian cycle. Cycles in FRQ levels at different temperatures are shown in green. A step up in temperature at any time (red arrows) results in not enough FRQ to close the feedback loop, so the clock is reset to the time corresponding to low FRQ, subjective dawn. A step down in temperature at any time leaves the clock with sufficient FRQ to close the feedback loop; the cycle is reset to the time marked by high FRQ, around subjective evening. Adapted from reference 24.

There is a reliable daily rhythm in body temperature with an amplitude of greater than 1°C; cycles of this magnitude are sufficient to entrain the clocks of cells in culture (81) and probably contribute to keeping cells in synchrony within our bodies.

The molecular mechanisms underlying temperature entrainment were first examined in *Neurospora* and the molecular response appears distinct from that seen for light (Fig. 6). In *Neurospora*, the amount of *frq* mRNA is not regulated by temperature and remains fairly constant across the physiological range; however, posttranscriptional mechanisms govern the amount of total FRQ made such that FRQ cycles around a higher mean level at higher temperatures (82). Even the lowest amount of total cellular FRQ seen at 30°C is higher than the highest amount seen at 20°C, and this fact drives the model for resetting based on temperature steps (83). When there is a step from high to low temperature, no matter at what time of day, the shift happens; there is so much FRQ that the clock is set to the time corresponding to peak FRQ levels, around subjective dusk. Conversely, following a shift from low to high temperatures, there is so little FRQ that the clock is set to the time of day corresponding to low FRQ levels, around subjective dawn (84).

OUTPUT FROM THE CLOCK

Circadian clocks are important because they control and coordinate the activities of cells so that appropriate biochemical activities happen at appropriate times of day. There are numerous examples from the circadian literature demonstrating the advantage of circadian regulation, chiefly by showing that organisms with clocks whose period lengths correspond with environmental

cycles outcompete organisms with clocks whose period lengths are different from the environment: for instance, wild-type clocks do better than long-period clocks in a 24-h environment, but long-period clocks do better than wild-type clocks in a long-period environment (e.g., reference 85). There are a variety of reasons why this might be the case. For instance, organization and partition of metabolism by time of day would prevent futile cycles and ensure that necessary precursors are available for metabolic needs.

In *Neurospora*, as has been described earlier, there is a very obvious rhythm in growth and development. This is seen under light-dark cycles but not in constant darkness unless carbon sources are limited or efforts are made to reduce ambient CO_2 or to enhance levels of reactive oxygen species (86, 87). It follows that there should be a rhythm in gene expression that supports these visible outputs from the clock, and indeed there is, but it should be noted that this plain quest for clock-regulated genes was first formulated and pursued in *Neurospora* (88). In the late 1980s, efforts were undertaken to use subtractive hybridization to enrich for genes whose expression peaked in a time-of-day-specific manner and this resulted in the identification of *clock-controlled gene*-1 (*ccg*-1) and *ccg*-2 (later shown to be allelic to *eas* [89]). This work also introduced the term "clock-controlled gene" into the circadian lexicon where it is now commonly used: clock-controlled genes are genes whose daily expression is controlled in a circadian fashion by the clock, but whose gene products do not generally impact the operation of the clock (Fig. 7A). This distinction, although it seems obvious now, was truly novel at the time and laid the foundation for studying output as a distinct subfield with circadian research.

Circadian Control of Transcription

It is now widely recognized that the principal means of output from the clock in all organisms from bacteria through humans is through daily control of transcription and the generation of *ccg*s. Efforts to identify *ccg*s have tracked the available technologies through the years, beginning with subtractive hybridization, and then differential hybridization, expressed sequence tag (EST) sequencing, microarrays, and most recently (and successfully) RNA-seq, which is now taken as the state of the art. This technology has been applied to identify the *ccg*s in a wide variety of organisms including *Neurospora*.

Some technical issues surrounding the identification of *ccg*s should be mentioned. Overall gene expression will change in response to time-of-day in constant conditions (true circadianly regulated genes), but will also change in response to age of the culture, ambient temperature, changes in nutrition (e.g., a shift in nitrogen sources from ammonium to nitrate, or from glucose to cellulose, or a variety of other factors). Because of this, efforts must be made to avoid such changes, beginning with the use of a medium such as Bird (90) that contains only one nitrogen source. Cultures must be as close as possible to the same age (hours since germination) as is feasible, and, to address this, strict schedules have been developed to adjust the times of transfer from light to dark (the cue that sets the clock running) of sets of cultures so that when all are harvested they can be the same developmental age but represent different circadian clock times (88, 91). There have been steady developments in software to extract rhythmic signals from the nonrhythmic background; these are now highly developed, but, in passing, a few technical issues should be noted. There are more stringent (JTK [92, 93]) and less stringent (ARSER [94]) software and algorithms available for calling rhythmic genes. To be dependably able to call rhythmic genes, sampling density must be high; samples taken at least every 2 h over 2 days are needed to reliably identify even 80% of rhythmic genes, and hourly sampling is better (92). Last, time series data can be highly influenced by initial conditions, and, in *Neurospora*, the first hours are still strongly influenced by the previous light-dark cycle; in no case can data from a single day be concatenated to make an imaginary two-day time series unless the goal is imaginary data.

Early studies (e.g., references 88 and 95) identified *ccg*s, but, because of their inherent limitations, tended to identify only the most abundantly expressed genes, and these were numbered in the order they were identified from *ccg*-1 to *ccg*-16. At this point, there was the realization that there would be potentially thousands of *ccg*s (as there now are) and so this method of naming was discontinued (96, 97). The most recent data that adhere to the standards of the previous paragraph are shown in Fig. 7B. These data were collected from cultures growing in liquid medium with glucose and gentle shaking in constant darkness. Under these conditions, there will be only very limited development (no aerial hyphae, for instance) so the spectrum of *ccg*s might be greatly expanded if cultures were collected from solid medium. Nonetheless and despite the relatively restricted overt development, the data show that as much as 40% of the genome is clock controlled (false discovery rate of 0.05) and roughly 10% is strongly and dependably clock controlled (91). This is likely to be an underestimate because, when random promoters were

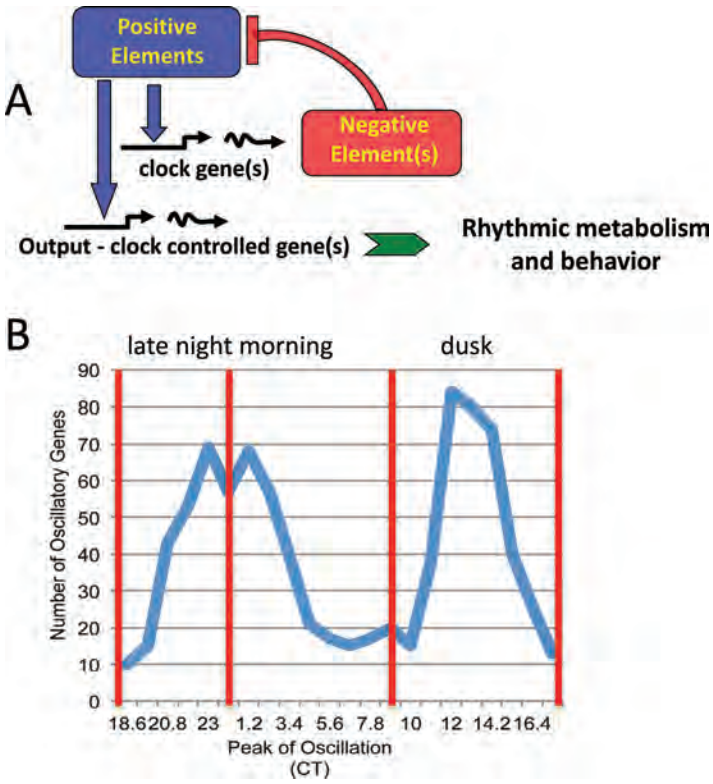


Figure 7 Output from the circadian clock. (**A**) The relationship of output to the oscillator is shown. The circadian clock is fungi and animals can be approximated as a single-step negative feedback loop in which the Positive Elements (in blue, the heterodimer of WC-1 and WC-2 or in animals BMAL1 and CLOCK) drive expression of the gene(s) encoding the Negative Elements (in red). The Negative Elements feed back, physically interact with the Positive Elements, and turn down their activity. The cycle thus generates rhythmic activity of the Positive Elements. When they, in turn, regulate genes whose products do not participate in the feedback loop, there are the means for transcriptional output from the clock. These are the *ccg*s. Adapted from reference 58. (**B**) The abundance of *ccg*s is shown as a function of the time of day at which their levels peak. The data are from 2-h bins across the day, with a bimodal peak of gene expression in the late night to morning and another peak around dusk (from reference 91).

assayed for their ability to drive circadian gene expression by fusion with a luciferase reporter, 75% were shown to be rhythmic only by luciferase; these were predominantly low-abundance genes, suggesting that the depth of sequencing was underpowered to detect rhythms at a 0.05 confidence level when there were simply too few transcripts present. Another interesting conclusion is that the spectrum of *ccg*s changes with the composition of the growth medium. It is plain that circadian regulation exerts a profound influence on the genome, and, in fact, if the criteria for choosing *ccg*s are relaxed just a bit, the majority of expressed genes will show some influence of time of day.

This finding has several important implications. First, it is plain that culture conditions matter a great deal in all experiments and not just if one is looking for *ccg*s. Prior light-dark treatment, as well as light-dark conditions during an experiment, can manifestly influence what is and is not seen. The level of expression of a gene might appear to be 10-fold higher or lower depending on when tissue is collected from an incubator that excludes light. Sometimes effects are due to circadian regulation but sometimes it is just light. An interesting example is the identification of DNA methylation at several places in the genome including the *frq* locus; methylation is plainly seen if cultures have been exposed to light but not if they have been in extended darkness or at elevated temperatures (33). There are also implications simply for choosing invariant "control genes" for normalization of reverse transcriptase-

quantitative PCR data, because many genes, including those commonly used as "invariant controls" such as *gapdh* (also known as *ccg*-7), are in fact clock controlled. In a useful spin-off from this study, mathematical tools were developed and used to identify truly invariant genes whose expression levels span the range of normal gene expression (98). The genes listed in this study provide truly valid control genes for normalization of gene expression studies.

The data of Hurley et al. (91), and to some extent Sancar et al. (although, because of the experimental design, these data are strongly influenced by the prior light-dark cycle [99]), reveal significant metabolic partitioning as a function of time of day that extends to control of major aspects of cell biology including growth, protein synthesis, stress responses, and intermediary metabolism. The greatest influence of circadian control on gene expression is seen around subjective dawn and dusk and the fewest relative number of *ccg*s peak in the middle of the day and night. While a great amount of detail is known and could be written about this, it suffices to say here that, in broad outline, morning and daytime gene expression tends to favor catabolism, energy production, and precursor assembly, whereas dusk and nighttime gene expression tends to favor genes whose products are involved in biosynthesis of cellular components and biomass accumulation (91).

The timing of transcriptional regulation itself provides abundant means for regulation. Whenever the WCC drives expression of another transcription factor (TF), regulation of that TF can provide a means for altering phase because it can feed back or forward to influence other TFs and coregulators, and, in addition, the TF itself can be posttranslationally modified. A current edge of research is the description of this network of TFs that drives their expression and other TFs that they in turn regulate.

After the first tier of transcriptional regulation, of course, a number of additional steps are involved in filtering the output from the clock. This is most clearly understood when one realizes that the circadian signal begins with the rhythmic activation of the WCC that, based on the expression of *frq*, its most salient output, peaks in the morning. It follows that, in order to obtain the spectrum of peak output phases shown in Fig. 7B, there must be many additional levels of regulation. We can begin with the act of transcription yielding a nascent transcript. Splicing of the transcript can be regulated followed by transcript stability. As a rule of thumb, as the stability of a transcript increases, its peak phase will become more and more delayed compared with the peak time of it transcription. Hurley et al. attempted to estimate regulation at the posttranscriptional level by comparing the peak times of *ccg*s from RNA-seq (i.e., total amounts) with the peaks times of expression based on promoter luciferase fusions for around 300 genes (91). The two did not match well for a large number of genes, indicating that additional levels of posttranscriptional regulation of transcripts are not uncommon.

Moving down the Central Dogma from mRNA there can be regulation at the level of translation, posttranslational modification of the protein to influence its binding partners, activity, or intracellular localization, and finally to regulate its stability. Many of these levels of regulation also have the capacity for global effects. For instance, another edge of research in *Neurospora* is regulation of kinase activity. Roles for mitogen-activated protein kinases (MAPK) in cellular responses to stress or in mating are well known (e.g., reference 100) and they activate a wide variety of proteins, but interestingly the clock has been shown to regulate basal MAPK signaling (101, 102). In *Neurospora*, OS-4 is the MAPKKK that initiates signaling through the cascade in response to stress and OS-2 is the terminal effector kinase in the MAPK cascade. In the subjective morning, OS-2 activity is high both because the WCC directly drives high OS-4 levels and because the activity of the OS-4 inhibitor RRG-1 is low, again due to circadian regulation. In the evening, the reverse is true. Circadian regulation of MAPK signaling is another aspect of regulation conserved between *Neurospora* and mammals (103).

NONCIRCADIAN OSCILLATORS IN FUNGI

Most fungi will display discernible rhythms in growth, morphology, or development under light-dark and temperature cycles, but only a few of these are actually circadian in nature. The "rhythms" appear because most fungi, with the exception of yeasts, can perceive and respond to light, as described in the review by Fischer et al. (129), and temperature is an all-pervasive metabolic controller. However, as noted earlier, under the right conditions—even conditions of constant nutrition, darkness, temperature, and humidity—a wide variety of fungi will express rhythms in growth habit, growth rate, hyphal branching, morphology, any or all of which are likely accompanied by changes in gene expression. These rhythms no doubt contribute to the vitality of the organism and they received much attention in the 1960s and 1970s; however, as the definition of circadian was refined, it became clear that they are not truly circadian (3). This was an important stage in the development of circadian biology as a field, because it was only when

such diverse systems were jettisoned that it became possible to focus on commonalities in approach and mechanism. As every geneticist knows, screens are possible only when phenotypes are robust, so it was this winnowing of noncircadian from truly circadian rhythms (e.g., 22, 104) that allowed the field to focus and deliver a molecular answer to the question of how the circadian clock works. The first screens for clock mutants were initiated by Jerry Feldman in the early 1970s (105) and yielded the *frq* gene whose cloning in the early 1980s (106) nucleated the molecular dissection of the clock in *Neurospora*.

Leaving circadian rhythms aside for the present, there is a very rich biology describing noncircadian rhythms, although much of it is now becoming dated. Noncircadian rhythms in hyphal branching or zonation are known in *Podospora anserina* (107), *Aspergillus niger* (108), *Leptosphaeria michotii* (e.g., 109), *Nectria cinnabarina* (110), along with *Penicillium* spp., *Alternaria tenuis*, and a number of additional fungi (3, 111). There is even a rhythm in the formation of nematode traps by the nematophagous fungus, *Arthrobotrys oligospora* (112). The first noncircadian rhythms in *N. crassa* date from the 1960s (113), and more than 3 decades ago, the defining FLO rhythm was described in the *frq*-null strain frq^9 (114, 115). When *frq* was cloned and the mutant alleles analyzed, the allele was shown to encode a truncated and nonfunctional FRQ, and the existence of the noncircadian oscillator was confirmed (68); it can have a period between 12 and 34 h depending on the nutrition and temperature (that is, it is completely uncompensated) and cannot be entrained by light. Since then, in *Neurospora* where cellular rhythms have been studied more deeply than in any other cell, there are at least nine noncircadian rhythms described that can be distinguished by their period lengths or the means used to induce them (e.g., 96, 116, 117–121). Generically, these are termed FLOs (for FRQ-less oscillators [122]). All share the property of cyclicity displaying in the aggregate periods ranging from 10 to over 100 h, and none show the full range of defining circadian characteristics.

Much confusion in the field, and within fungal biology, has arisen from investigators confusing the output of the circadian clock with the clock itself. In *Neurospora*, the clock controls conidiation as seen on race tubes. The logical fallacy has been to assume that anything that controls conidiation must therefore be part of the circadian clock. This statement is obviously flawed because conidiation, like any developmental process, can be controlled by many factors and will not always happen; it is merely one obvious aspect of overt circadian control along with all of the rhythms in gene expression noted above. Although there are detailed descriptions of many FLOs, to date it is still unclear whether these serve any adaptive function to the organism or whether they are simply accidental by-products of metabolic regulation by feedback. On this point, the NRO oscillator, which appears to derive from feedback loops associated with regulation of nitrate reductase, is instructive (116). In part because of the logical fallacy of equating all conidiation rhythms with circadian rhythms, some FLOs have been studied in depth in the hope that they would reveal something of use for understanding circadian rhythms, and one that has been studied most intensively is induced by choline starvation (119). This CDO (choline deficiency oscillator) exerts control of overt growth and development such that conidiation rhythmicity can assume a period well in excess of 100 h, and growth is up to 8-fold faster between conidial bands than within conidial bands. The CDO is not temperature compensated, has lost phase control, and is light-sensitive in $wc\text{-}1^+$ genetic backgrounds and (obviously) does not require *frq* (123). The question of whether this oscillator interacts with the circadian FRQ/WC oscillator was answered definitively in experiments using a luciferase reporter to track *frq* expression and WCC activity under conditions of choline limitation where the CDO had taken control of conidiation. These experiments, done in *frq*+ and *frq* [period mutant] backgrounds, clearly showed the circadian FRQ/WC oscillator running in the background with a period appropriate to the *frq* allele (22 or 29 h), while the CDO ran with a ca. 80-h period length. The two cycles crossed each other at all phases more than once with no evidence for interaction (124).

Although the CDO plainly does not interact with the circadian clock, other FLOs might take input from the clock, in particular, those with period lengths comparable to the circadian clock, i.e., circa 24 h, like the NRO but unlike the CDO. In this context, one rationale for the existence of FLOs is that they might serve as slave oscillators that could under the right conditions assume proximal control of aspects of metabolism while remaining under the overall control of the circadian system. A slave oscillator could be open to evolutionary fine-tuning without altering the more finely tuned compensated/entrainable circadian clock, and the slave need not have the full complement of circadian properties; in a normal cell it would still be a part of the circadian system as it would be entrained by the circadian clock. In a *frq* or *wc* null mutant background and absent the circadian clock, each slave reverts to its inherent periodicity (28, 29, 125, 126).

CONCLUSION

Neurospora has provided a durable system for the study of biological clocks and oscillators in the broadest sense, and more specifically in circadian rhythms. Decades of analysis have shown that the *Neurospora* circadian clocks share a common regulatory architecture as well as conserved components with circadian oscillators in animals, and many aspects of circadian biology and molecular biology in these organisms were first elucidated in *Neurospora*. These include the first systematic screens for *ccg*s and elucidation of transcriptional control as a principal means for circadian output (88), experimental proof that transcriptional negative feedback is at the core of the clock (68), elucidation of the mechanism for light-induced (70) and temperature-induced (83) clock resetting, identification of the first PAS domain-mediated heterodimer as the positive element in a circadian feedback loop (127), and closure of the loop in a single step via action of the negative element on the positive element (127). Work on *Neurospora* also led the way to understanding the cellular roles of noncircadian oscillators and documented the fact that many such oscillators can coexist within a single mycelium. Many of the components involved in both kinds of oscillators are conserved among fungi and even more broadly.

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Target of Rapamycin (TOR) Regulates Growth in Response to Nutritional Signals

25

Ronit Weisman

INTRODUCTION

A universal feature of all organisms is their ability to respond to nutrient availability by regulating growth and developmental programs. The identification of the target of rapamycin (TOR) pathway was a seminal discovery in the quest to understand the molecular mechanisms that govern such processes. TOR is an evolutionarily conserved serine/threonine kinase belonging to the family of phosphatidylinositol kinase-related kinases. Other members of this family include the mammalian DNA damage checkpoint kinases ATM and ATR,which are conserved from yeast to human, and DNA-PK and SMG1, which are not found in yeasts. TOR regulates growth (accumulation of mass), proliferation (accumulation in cell number), and survival in response to nutritional changes by diverse mechanisms that include regulation of anabolic and catabolic metabolism, nutrient uptake, protein translation and turnover, gene transcription, and the epigenome (reviewed in 1–3).

The first TOR genes were isolated in the budding yeast *Saccharomyces cerevisiae* as point mutations that conferred resistance to the growth-inhibiting effect of rapamycin (4–6). Rapamycin is a small hydrophobic molecule, a product of the soil bacterium *Streptomyces hygroscopicus* that was first isolated as an antifungal antibiotic but later proved to be a potent inhibitor of cellular growth in humans via inhibition of mammalian TOR (mTOR). The antiproliferative effect of rapamycin has proved beneficial for several clinical applications, including immunosuppression for the prevention of allograft rejection, treatment of cancer, and elution of stents to prevent restenosis after angioplasty (7, 8). Rapamycin induces cell growth arrest by mimicking a starvation-like response, which is characterized, among other features, by the induction of autophagy. This feature of rapamycin and its derivatives, or second-generation ATP-competitive TOR-specific inhibitors, may prove useful for the treatment of neurodegenerative and metabolic diseases (7, 8). Rapamycin, somewhat similar to calorie restriction, extends the life span in several model systems, including yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice, making it the first drug with the potential to treat aging (7, 8).

TOR proteins can be found in two distinct evolutionarily conserved complexes termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (see Fig. 1). TOR provides the catalytic subunit in these complexes and is accompanied by TORC1-specific and TORC2-specific subunits, as well as protein subunits that are shared by the two complexes. TORC1 and TORC2 are controlled by distinct upstream and downstream regulators but share common features, such as the phosphorylation and activation of AGC kinases (a family of protein kinases named after protein kinases A, G, and C) at their C terminal hydrophobic and turn motifs. Since distinct AGC kinases are phosphorylated and activated by TORC1 and TORC2, monitoring the phosphorylation status of these kinases at specific sites is most useful for determining TORC1- or TORC2-specific activation (1).

In higher eukaryotes (nematodes, flies, mice, and humans) there is a single gene for TOR. In contrast,

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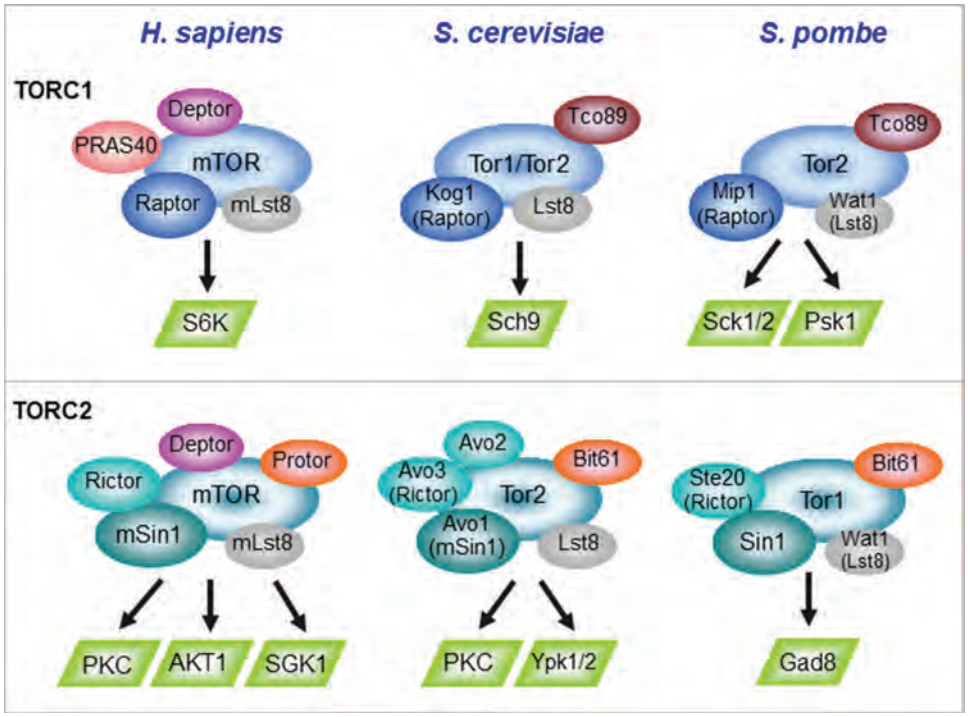


Figure 1 TORC1 and TORC2 subunits and downstream AGC kinases in mammalian, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* cells. TORC1 and TORC2 have shared and unique components. The human protein Raptor is TORC1-specific and is conserved in both yeast species. The human proteins Rictor and Sin1 are TORC2-specific subunits and are conserved in both yeast species. The target kinases of TORC1 and TORC2 are shown as green parallelograms.

two TOR genes are found in several fungal groups as the result of independent events of whole genome or segmental duplications (9). The two best-studied unicellular organisms for TOR signaling are the budding yeast *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, which contain two TOR genes. In *S. cerevisiae*, TORC1 (*Sc*TORC1) mainly contains *Sc*Tor1 as the catalytic subunit, but a minor *Sc*TORC1 variant that contains *Sc*Tor2 also exists; ScTORC2 contains *Sc*Tor2 as the catalytic subunit (10). In *S. pombe*, *Sp*Tor2 mainly acts as the catalytic subunit of *S. pombe* TORC1 (*Sp*TORC1), while *Sp*Tor1 mainly acts as the catalytic subunit of *Sp*TORC2 (11, 12). This awkward naming of the *S. pombe* TOR genes is the result of their identification prior to the isolation of the TOR complexes in *S. cerevisiae* (13) and reflects the lack of known specific sequences for the TOR catalytic subunit that can predict its association with TORC1 or TORC2 subunits. A single TOR gene is found in *Candida albicans* and filamentous fungi, and TOR inhibition plays an important role in the pathogenicity of these organisms (14–16) (see further discussion below).

TORC1: A MASTER REGULATOR OF GROWTH AND STARVATION RESPONSES

TORC1 Architecture, Rapamycin Sensitivity, and Downstream Signaling

TORC1 is defined by the essential subunit known as Raptor in metazoans, Kog1 in *S. cerevisiae*, and Mip1 in *S. pombe*. A highly conserved but nonspecific subunit is Lst8 (also known as GβL in metazoans and Wat1 or Pop3 in *S. pombe*), which is found both in TORC1 and TORC2. Other components of TORC1 are not as highly conserved; Tco89 is found in *S. cerevisiae*, *S. pombe*, and *C. albicans*, but no homologues have been identified in higher eukaryotes. In contrast, PRAS40 and Deptor are found in higher eukaryotes, but no homologues have been identified in fungi (1) (see Fig. 1).

The TOR catalytic subunit in TORC1 binds rapamycin when the drug is in complex with the prolyl isomerase FKBP12. The FKBP12-rapamycin complex (hereafter referred to simply as rapamycin) binds the FKBP12-rapamycin binding (FRB) domain in TOR, which is located in close proximity to the kinase domain. As a

result, most (but not all) TORC1-dependent activities are inhibited. Studies of *S. cerevisiae* played a central role in understanding the mode of action of rapamycin, including the identification of FKBP12 and the FKBP12-rapamycin binding (FKBP12/rapamycin-binding) domain and the characterization of the differential sensitivity to rapamycin of TORC1 and TORC2 (1, 4–6, 10). Still, the mechanism by which rapamycin inhibits TOR signaling and the determinants that establish rapamycin sensitivity are not fully understood. Several mechanisms of action have been suggested, including rapamycin-mediated disruption of TOR complexes and inhibition of the access of specific substrates to the kinase domain of TOR (reviewed in 17). TORC2 is less sensitive to inhibition by rapamycin compared with TORC1 or is not sensitive at all. Recent structural studies of *Sc*TORC2 suggest that the *Sc*TORC2-specific subunit Avo3 (Rictor in metazoans) masks the rapamycin-FKBP12 binding site of Tor2, thus preventing the inhibition of *Sc*TORC2 signaling (18).

The specificity of rapamycin for TORC1 proved most useful in deciphering cellular functions of this complex. Disruption of TORC1 in *S. cerevisiae*, either through genetic manipulations or inhibition by rapamycin, results in a phenotype that resembles starved cells, including arrest at the G1 phase of the cell cycle, G0-like cellular morphology and physiology, rapid drop in protein synthesis, repression of ribosomal gene expression, induction of stress- and nutrient-starvation genes, and stimulation of autophagy (19–22). Further studies demonstrated a conserved role of TORC1 in growth regulation through stimulating nutrient uptake, ribosome biogenesis, and the synthesis of proteins, lipids, and nucleotides (23–25; reviewed in 1, 26). To methodically screen for conditions that activate *Sc*TORC1, the phosphorylation status of Sch9, the AGC kinase downstream of *Sc*TORC1, was monitored. These studies demonstrated that Sch9 becomes dephosphorylated and inactivated in response to nitrogen, carbon, or phosphate starvation; high temperature; salt or redox conditions (27); and amino acid starvation or low quality of the nitrogen source (28). However, the signal or signals that are transduced to *Sc*TORC1 under these conditions are only partially understood.

Somewhat curiously, the growth of wild-type *S. pombe* cells is resistant to treatment with rapamycin (29). Yet rapamycin inhibits the phosphorylation of Psk1, the AGC kinase that lies downstream of *Sp*TORC1, or the phosphorylation of the Rps6 protein (S6 in human), the substrate of Psk1 (30, 31). S6 is a well-established target for mTORC1-S6K in humans and is often used to screen for hyperactivation of mTOR in cancer cells (32). Since rapamycin does not inhibit the essential function of *Sp*TORC1, conditional mutants of the catalytic subunit of *Sp*TORC1, *tor2*, or *mip1* (Raptor homologue) are used to study the cellular functions of *Sp*TORC1. Disruption of *Sp*TORC1 resulted in a phenotype that specifically resembles nitrogen-starved cells: growth arrest at the G1 phase of the cell cycle (unlike growth arrest at the G2 phase, characteristic for carbon starvation in *S. pombe*), small and rounded cell morphology, induction of nitrogen-starvation-induced genes, and activation of the sexual development pathway (11, 12, 33, 34). Complete withdrawal of the nitrogen source from the medium or a shift to a poor-quality nitrogen source (proline instead of ammonium) resulted in downregulation of *Sp*TOC1 activity as measured by decreased Rps6 phosphorylation (30, 35), leading to division at a reduced size and advancement into mitosis (36, 37). In *C. albicans*, rapamycin inhibits growth via inhibition of the single TOR gene, *Tor1*, while nitrogen and carbon starvation both result in reduced *Ca*S6 phosphorylation (15, 38), suggesting that similar to *S. cerevisiae*, *Ca*TORC1 is sensitive to rapamycin and responds to both carbon and nitrogen limitation.

At the transcriptional level, inhibition of *Sc*TORC1 or rapamycin treatment in *S. cerevisiae* (20, 21, 39, 40), disruption of *Sp*TORC1 (12, 33), or rapamycin treatment in *C. albicans* (41) led to the reduction of ribosomal gene expression and induction of starvation- or stress-responsive transcription. Of note is the regulation of the response to changes in quantity or quality of the nitrogen source by the conserved TORC1-protein phosphatase 2A (PP2A) module, which regulates the activity of GATA-transcription factors (a family of transcription factors that are characterized by their ability to bind DNA sequences that contain the GATA motif). Early studies identified the zinc-finger GATA transcription factors Gln3 and Gat1 as targets for *Sc*TORC1 (20, 21, 40). Further studies unraveled a pathway in which *Sc*TORC1 regulates the activity of PP2A and PP2A-like phosphatases such as Sit4 by regulating their association with the inhibitory regulatory subunit Tap42. Upon reduction of the quantity or quality of the nitrogen source, *Sc*TORC1 is inactivated, promoting the disassociation of Tap42 and the activation of the phosphatases. In turn, the Ure2 protein that sequesters Gln3 and Gat1 is dephosphorylated, and Gln3 and Gat1 are dephosphorylated and translocate into the nucleus (20, 21, 42–44). Gln3 and Gat1 are required for transcriptional activation of genes that are normally repressed in the presence of high quantity and quality of the nitrogen source and are critical regulators of nitrogen

catabolite repression, the processes by which high-quality nitrogen sources are imported and assimilated in preference to poor-quality nitrogen sources.

The *C. albicans GLN3* and *GAT1* homologues are also required for regulation of nitrogen metabolism, and their disruption resulted in rapamycin resistance, similar to the effect of disrupting *GLN3* and *GAT1* in *S. cerevisiae* (45). More recently, *Sp*TORC1 was demonstrated to regulate the phosphorylation and nuclear localization of the GATA transcription factor Gaf1. Gaf1 becomes dephosphorylated and inactivated in a manner dependent on the PP2A phosphatase Ppe1 in response to nitrogen (but not carbon) starvation (46, 47). The conservation of the TORC1-PP2A-GATA-transcription signaling in the distantly related *S. cerevisiae* and *S. pombe*, two yeasts that diverged in evolution more than 300 million years ago, suggests that this pathway may also be conserved in higher eukaryotes.

Early studies showed that treatment of *S. cerevisiae* with rapamycin led to a rapid drop in protein synthesis (19). Further studies revealed that *Sc*TORC1-Sch9 and *Sc*TORC1-Tap42-PP2A play prominent roles in the regulation of ribosome biogenesis, one of the most energetically demanding processes and thus a focal point for the coordination of growth with nutritional changes. *Sc*TORC1 regulates ribosome biogenesis at many levels, including transcriptional induction of rRNA, ribosomal proteins, and the ribosome biogenesis (Ribi) regulon. Several transcription factors were identified as targets of *Sc*TORC1 in the regulation of ribosome biogenesis, including Maf1, the repressor of RNA pol III (23, 48), Sfp1 (49), the forkhead transcription factor Fhl1 (50), and the transcriptional repressors Stb3, Dot6, and Tod6 (24). *Sc*TORC1 also directly binds 35S rDNA promoters to promote rRNA synthesis (51). Similar to *S. cerevisiae*, *Sp*TORC1 also regulates transcription of ribosomal proteins (33) and phosphorylates the repressor of RNA pol III Maf1 (52). At the posttranscriptional level, *Sc*TORC1 regulates protein synthesis through regulation of the translation-initiation factor eIF2α, in a manner that is at least partially conserved in *S. pombe* (53, 54). In both *S. cerevisiae* and *S. pombe* eIF2α is phosphorylated in response to amino acid starvation or rapamycin treatment. The phosphorylation of eIF2α is mediated via the *S. cerevisiae* or *S. pombe* Gcn2 kinases; however, *Sc*TORC1 regulates the phosphorylation of Gcn2 at sites that are not conserved in *S. pombe*.

Autophagy is a general name for catabolic processes mediated by membrane trafficking pathways that lead cytoplasmic material from the cytosol into the lumen of the lysosome or vacuole, where massive degradation takes place. *Sc*TORC1 inhibits autophagy under favorable growth conditions through the phosphorylation of Atg13 that inhibits the assembly of the Atg1 protein kinase complex (55). Autophagy has been linked to several human pathologies, including cancer and neurodegenerative diseases and is also implicated in the process of aging. Most interestingly, rapamycin treatment or reduced TORC1 activity in *S. cerevisiae*, in *S. pombe*, or in higher eukaryotes extended the life span (56–58). TORC1 controls aging through Sch9 in *S. cerevisiae* (56), in a process that involves the induction of autophagy and stress genes, as reviewed in more detail in reference 8. Additional downstream effects of TORC1 have been studied in detail, including amino acid uptake, regulation of cell size, and developmental responses, including entrance into quiescence or regulation of filamentous growth. These are not detailed here due to limited space but are reviewed elsewhere (1, 59).

TORC1 Upstream Signaling and the Response to Nitrogen Quantity or Quality or the Presence of Amino Acids

In yeast cells, the activity of TORC1 is sensitive to nutritional starvation and a variety of stresses, while mTORC1 is also sensitive to energy levels (ATP) and growth factors (3). The direct signals or mechanisms for activation of TORC1 are as yet unknown; however, a large body of evidence suggests that the nitrogen source and/or amino acids (which may also serve as a nitrogen source) play a key role in TORC1 activation.

Nitrogen is an essential element required for synthesis of amino acids, nucleotides, and other cellular components. Yeast cells can sense, take up, and assimilate several nitrogen sources. A high-quality nitrogen source (glutamine or ammonium) is defined by its ability to promote rapid growth and suppress the nitrogen catabolite repression genes (60). As described above, *Sc*TORC1 and *Sp*TORC1 are major regulators of the transcriptional control in response to the quality and quantity of the nitrogen source, and the activity of these complexes drops in response to low levels or poor quality of the nitrogen source (28, 30, 35). *Sc*TORC1 and *Sp*TORC1 are also activated in response to specific amino acids such as leucine, glutamine, asparagine, arginine, aspartate, methionine, and cysteine (28, 61–64), raising the intriguing possibility that the nitrogenous compounds critical for TORC1 activation are amino acids. This possibility is particularly tempting in view that in higher eukaryotes, amino acids (particularly leucine, glutamine, and arginine) are potent activators of mTOR signaling (65–67). Whether amino acid levels are directly sensed by TORC1 and, if so, by what mechanisms is

still an open question. Moreover, the mechanism of activation of TORC1 by the quantity and quality of the nitrogen source appears distinct from the mechanism of response to amino acids (28, 36). Moreover, different amino acids are thought to activate TORC1 through different mechanisms, including glutaminolysis and leucyl-tRNA synthetase (61, 68).

TORC1 and several remarkably conserved upstream regulators of TORC1 are localized to the vacuole in yeast cells and to its equivalent compartment, the lysosome, in higher eukaryotes (reviewed in 17). At the vacuolar or lysosomal surface TORC1 is activated by two distinct guanosine triphosphate GTPases: the Gtr (Rag in higher eukaryotes) and the Rhb1 (Rheb in higher eukaryotes). The Rag/Gtr-TORC1 axis is conserved in *S. cerevisiae* and *S. pombe*, while the Rhb1/Rheb-TORC1 axis is conserved in *S. pombe* but is absent in *S. cerevisiae* (see Fig. 2). The vacuole and lysosome act as major sites for protein degradation and reservoirs of amino acids; however, whether TORC1 responds to amino acids in the vacuole/lysosome or in the cytoplasm is not known yet.

In *S. cerevisiae*, the Gtr complex is composed of Gtr1 and Gtr2. Gtr1 is analogous to the mammalian RagA or RagB, while Gtr2 is analogous to RagC or RagD. Only when Gtr1 or RagA/B is bound to GTP and Gtr2 or RagC/D is bound to GDP is the heterodimer active and able to lead to activation of TORC1

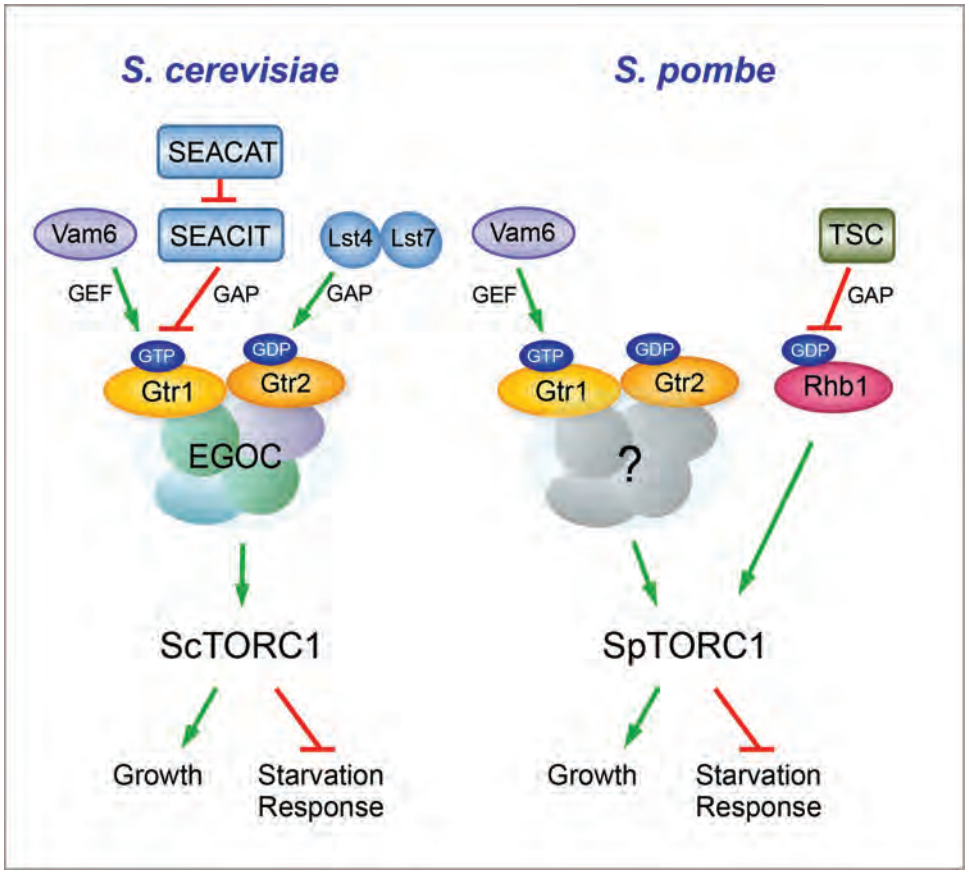


Figure 2 TORC1 is activated by GTPases to promote growth and inhibit starvation responses. *Saccharomyces cerevisiae* TORC1 (*Sc*TORC1) and *Schizosaccharomyces pombe* TORC1 (*Sp*TORC1) are activated by the GTPase complex Gtr1/Gtr2. The complex is active when Gtr1 is bound to GTP and Gtr2 is bound to GDP. Vam6 is a guanine exchange factor (GEF) for Gtr1 that is conserved between *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, the Gtr1/Gtr2 complex is associated with the EGO complex and is controlled by the SEACIT (which acts as GTPase activating protein, GAP), SEACAT, and Lst4-Lst7 complexes. These complexes have as yet unidentified equivalents in *S. pombe*. *Sp*TORC1 is also regulated by the Rhb1 GTPase (Rheb in mammals) and the TSC (tuberous sclerosis complex, a tumor suppressor complex in mammals), which acts as a GAP towards Rhb1.

(69–71). The Gtr1/2 GTPases associate with the EGO complex, which contains Ego1, Ego2, and Ego3 to form a complex that tethers Gtr1/2 and *Sc*TORC1 to the vacuolar membrane (71–73). The EGO equivalent complex in higher eukaryotes is the pentameric Ragulator complex, which is required for lysosmal localization and also acts as a guanine exchange factor toward RagA/B. In *S. cerevisiae* the guanine exchange factor activity toward Gtr1 is provided by Vam6 (vacuolar morphogenesis protein), which apparently has no mammalian equivalent (reviewed in 17). The vacuolar Vam6-Gtr1/2 module is conserved in *S. pombe* and activates *Sp*TORC1 in response to the presence of amino acids (62). The interaction and activation of either *Sc*TORC1 or *Sp*TORC1 by the Vam6-Gtr1/2 module are sensitive to the presence of amino acids, but *Sc*TORC1 and *Sp*TORC1 are localized to the vacuole irrespective of the nutritional status (62, 71). In contrast, mTORC1 dissociates from the lysosome upon amino acid starvation (69, 70). The Gtr and Rag complexes are negatively regulated by conserved GAP (GTPase activating protein) complexes that are named SEACIT and GATOR1 in *S. cerevisiae* and humans, respectively (Fig. 2). SEACIT and GATOR1 are in turn subjected to negative regulation by additional conserved complexes, SEACAT and GATOR2 (74; reviewed in 17) (Fig. 2). More recently, Lst4-Lst7 was identified as the complex that acts as a GAP toward Gtr2, promoting the binding of GDP to Gtr2 and thus activating *Sc*TORC1 (64), similar to the FNIP-folliculin complex in mammalian cells (reviewed in 17).

The *S. cerevisiae* Gtr1/2 GTPases regulate TORC1 activity but are not essential genes, suggesting that they play only a limited role in activation of *Sc*TORC1. Similarly, disruption of *gtr1/2* in *S. pombe* leads to a reduced level of *Sp*TORC1 activity and a hyper-mating phenotype, characteristic of conditional loss-of-function *Sp*TORC1 mutants; still, *S. pombe* cells lacking Gtr1/2 or Vam6 can respond to changes in the nitrogen quantity or quality. These findings argue that the Gtr-TOR axis plays only a relatively limited role in activation of TORC1 (62).

While the Gtr1/2 complex is dispensable for activation of TORC1 in yeast cells, the *S. pombe* Rhb1 GTPase (Rheb in human) is essential for *Sp*TORC1 activation (75–78). Accordingly, loss of function of Rhb1 results in a phenotype that mimics nitrogen starvation and is highly similar to disruption of *Sp*TORC1, including the G1 arrest and activation of nitrogen-starvation gene expression. Rhb1 is negatively regulated by the TSC1-TSC2 complex, which is conserved in humans and functions as a GAP toward Rhb1. The TSC complex functions as a tumor suppressor complex, and mutations in either TSC1 or TSC2 can lead to the tuberous sclerosis syndrome, which is characterized by rapid development of tumors and severe neurological defects (79). Disruption of the TSC complex in *S. pombe* results in prolonged phosphorylation of ribosomal S6 under nitrogen starvation (31), reduced transcription of nitrogen-starvation-induced amino acid permeases, and reduced amino acid uptake (80, 81). These findings suggest that deletion of TSC in *S. pombe* leads to hyperactivation of *Sp*TORC1 that shuts down transcriptional programs dedicated to scavenging for nitrogen sources. *C. albicans* homologues for Rheb and TSC2 were also identified. Disruption of *Ca*Rhb1 resulted in hypersensitivity to rapamycin, while *Ca*Rhb1 overexpression and *Ca*Tsc2 deletion are defective in filamentous growth under low or poor nitrogen, suggesting that Rhb1 activates *Ca*TORC1 and prevents normal responses to nitrogen starvation (82).

The TSC-Rheb axis acts as a hub for several signaling pathways, among them the AMPK, a serine/threonine kinase that coordinates cell growth and metabolism with available energy resources (reviewed in 83). AMPK is a heterotrimeric complex composed of a catalytic (α) subunit and two regulatory (β and γ) subunits. In humans, the tumor suppressors LKB1 and CaMKK (calcium/calmodulin-dependent protein kinase kinase) activate mammalian AMPK, which in turn directly phosphorylates TSC2 and activates its GAP activity, leading to conversion of Rheb to its inactive form and inhibition of mTORC1 (84; reviewed in reference 83). It was recently demonstrated that in *S. pombe*, the α subunit of AMPK is required to respond to changes in the quality of the nitrogen source. Under nitrogen stress conditions (shift from a high-quality to poor-quality nitrogen source), a specific homologue of CaMKK (Ppk34) stimulates *Sp*AMPK activity, which leads to downregulation of *Sp*TORC1 in a manner dependent on the TSC-Rhb1 (36). How TORC1 may integrate the complex signals from different pathways and coordinate these with the nutritional status awaits further studies.

TORC2: ROLES IN CELLULAR METABOLISM, GROWTH, AND SURVIVAL

TORC2 Architecture and Downstream Signaling

TORC2 contains the TOR catalytic subunit together with two additional highly conserved proteins that are essential for the function of the complex: Rictor

in metazoans (Avo3 in *S. cerevisiae* and Ste20 in *S. pombe*) and mSin1 in metazoans (Avo1 in *S. cerevisiae* and Sin1 in *S. pombe*) (see Fig. 1). TORC2 contains the Lst8 subunit, which is also found in TORC1. The Protor subunit in mammals has orthologues in *S. cerevisiae* and *S. pombe* (Bit61). Avo2 is specific for *S. cerevisiae* and *C. albicans*, while Deptor (which is also part of mTORC1) has been identified only in mammals (reviewed in 1).

The cellular functions of TORC2 are less well understood compared with TORC1. This is partly due to the lack of TORC2-specific inhibitors. In addition, in contrast to the starvation-like phenotypes observed upon disruption of TORC1, the loss of TORC2 results in diverse effects (reviewed in 85), making it difficult to attribute a primary cellular function to TORC2. Recent findings suggest that TORC2 plays significant roles in cellular metabolism, including cancer metabolism, growth control, and survival, encouraging further research of TORC2 and the search for TORC2-specific inhibitors (85, 86). As in TORC1 signaling, AGC kinases downstream of TORC2 play important roles in mediating downstream effects (see Fig. 1). The best-studied AGC kinases downstream of *Sc*TORC2 are Ypk1 and Ypk2 (87), while most of *Sp*TORC2 functions are mediated via Gad8 (88).

*Sc*TORC2 is essential for growth, while *Sp*TORC2 becomes essential only under starvation and a variety of other stress conditions. So far, only a limited overlap exists between the functions attributed to *Sc*TORC2 and *Sp*TORC2. It is not clear whether this reflects a true divergence in function or our incomplete understanding of the cellular roles of TORC2. *Sc*TORC2 was first implicated in the regulation of the polarization of the actin cytoskeleton, suggesting that it mainly regulates spatial aspects of cell growth (89, 90). Further studies revealed that *Sc*TORC2-Ypk1/2 also controls endocytosis (91) and sphingolipid biosynthesis (92–95), in a manner that is antagonized by the calcium- and calmodulin-dependent protein phosphatase calcineurin, which acts either directly downstream of *Sc*TORC2 or in a parallel pathway (92, 93). *Sc*TORC2 regulates actin polarization and endocytosis via flipase protein kinases (96–99), in a manner that was also suggested to be linked to the control of reactive oxygen species accumulation (97, 98). Interestingly, the level of reactive oxygen species is regulated by *Sc*TORC2 and can act as a signal to activate *Sc*TORC2 (97, 98). The Slm proteins, which were first identified downstream of *Sc*TORC2, can also function upstream of *Sc*TORC2 to promote activation of this complex in response to plasma membrane stress, resulting in a homeostatic loop in which *Sc*TORC2 regulates several aspects of membrane biogenesis and is also affected by changes in the membrane (100). Recently, it was demonstrated that *Sc*TORC2-Ypk1 positively regulates autophagy in response to amino acid starvation (101). This finding is interesting because it suggests that *Sc*TORC1 and *Sc*TORC2 oppositely regulate autophagy, albeit by different mechanisms. It may also suggest that *Sc*TORC2 is linked to nutritional changes more closely than has previously been recognized.

The most pronounced effects of loss of function of *Sp*TORC2-Gad8 become apparent under starvation and stress conditions. *Sp*TORC2 is essential to execute the two main responses to starvation: sexual development and entrance into stationary phase. Thus, cells that lack *Sp*TORC2 or Gad8 are highly sterile and quickly die once they exit the logarithmic phase (13, 102). Loss of *Sp*TORC2-Gad8 also renders cells sensitive to a variety of stress conditions, including low or high temperature, osmotic or oxidative stress, and DNA damage or replication stress conditions (13, 103, 104). Under normal growth conditions, *Sp*TORC2-Gad8 is required for the G2/M transition, and cells disrupted for *Sp*TORC2 show an elongated phenotype characteristic of a delay in entrance into mitosis (13, 103, 105, 106). *Sp*TORC2 is also required for amino acid uptake (107) and for the localization of Ght5, a high-affinity glucose transporter, to the cell surface under low-glucose conditions (108). An abnormal distribution of actin cortical dots and excess actin polymerization at the cell equator occur in cells lacking functional *Sp*TORC2 (106), as do defects in reorganizing the actin observed during new end take-off, the phase during which the new cell end starts to grow (109).

Unexpectedly, transcriptional profiles of *S. pombe* cells lacking the catalytic subunit of *Sp*TORC2 resembled those of cells lacking histone deacetylases or chromatin remodeling subunits (104). This finding led to the identification of additional defective phenotypes in *Sp*TORC2 mutant cells, including elongation of telomeres and loss of gene silencing (104). Of interest are the similarities observed upon loss of function of *Sc*TORC2 or *Sp*TORC2 with respect to DNA damage sensitivity (104, 110). In both cases, TORC2 plays a role in survival of DNA damage in a manner that is independent of DNA checkpoint activation but involves suppression of accumulation of DNA damage sites. However, in *S. cerevisiae*, elevated levels of DNA damage are thought to be the result of the defect in the actin cytoskeleton, whereas in *S. pombe* no such link is known (85). Interestingly, the inability of cells that lack *Sp*TORC2 or Gad8 to execute nitrogen starvation

responses is opposite to the "always starved for nitrogen" phenotype of cells lacking *Sp*TORC1 (35, 106). This is somewhat reminiscent of the opposite effects of *Sc*TORC1 and *Sc*TORC2 in the regulation of autophagy and may suggest regulatory links between *Sp*TORC1 and *Sp*TORC2.

TORC2 Upstream Signaling and the Response to Glucose Availability

The upstream regulation of TORC2 is poorly characterized. *Sc*TORC2 has been localized to membrane structure organelles, primarily to the plasma membrane, but has also been detected throughout the cytoplasm (111, 112). Visualization of GFP tagged *Sp*TORC2 components also indicated cytoplasmic as well as cortical localization (113), but a biochemical approach has suggested that Tor1, the catalytic subunit of *Sp*TORC2, and Gad8 are also found in the nucleus (114). *Sc*TORC2 is activated by direct association with the ribosome (115, 116), in a manner that is conserved in mammals and that may link TORC2 to growth capacity. In accord with the localization of *Sc*TORC2 to the plasma membrane, *Sc*TORC2 is activated by plasma membrane stress, which stems from cell surface expansion or stress on the plasma membrane (100). This mode of activation is thought to reflect a feedback loop mechanism in which *Sc*TORC2 regulates membrane biosynthesis but is also affected by membrane growth (17). In *S. pombe*, glucose but not nitrogen is required for activation of *Sp*TORC2 (117, 118). The activity of *Sp*TORC2-Gad8 in response to glucose requires the activation of Rhy1, a Rab family GTPase that lies upstream of *Sp*TORC2-Gad8 (118). The regulation of *Sp*TORC2-Gad8 in response to glucose availability is fast and does not require protein translation, suggesting a close link between a nutritional change and TORC2 activation, although the nature of the glucose signal that is sensed by *Sp*TORC2 is as yet unknown.

TOR IN PATHOGENIC FUNGI

Virulence in plant or human pathogenic fungi requires several cellular traits, including invasive growth, morphogenetic yeast-to-hyphal transition (e.g., in *C. albicans*), vegetative hyphal fusion, and expression of adhesion and stress response genes. As discussed above, TOR signaling regulates many aspects of the stress response as well as the switch from vegetative growth to developmental programs in response to environmental changes. Accordingly, accumulation of data indicates that TOR signaling is critical for virulence in several pathogenic fungi and may thus serve as a target for antifungal treatment. Early studies demonstrated that rapamycin and several less immunosuppressive analogues inhibited growth in *S. cerevisiae*, *C. albicans*, and *Cryptococcus neoformans*, suggesting that the use of rapamycin analogues may be beneficial as antifungal agents (15; reviewed in 16). Further support of the role of TOR in *C. albicans* pathogenicity is evident from studies that demonstrate the role of *Ca*Tor1 in regulating the expression of cell wall- and hyphal-specific genes, including adhesins (41). Moreover, genetic manipulation of *Ca*TOR signaling, including disruption of *Ca*Sch9 (119), *Ca*Rhb1 or *Ca*Tsc2 (82), or *Ca*Sit4 (120), supports a role for *Ca*Tor1 in promoting hyphal formation and virulence in response to nutritional changes, as well as in response to changes in pH (121). Interestingly, reduced *Ca*Tor1 signaling during hyphal initiation leads to the expression of the GATA transcription factor Brg1, which competes for promoter binding with Nrg1, the major transcription repressor of hyphal development (122). Thus, similar to *S. cerevisiae* or *S. pombe*, TOR is found to regulate a family member of the GATA transcription factors in *C. albicans*, but unlike *S. cerevisiae* or *S. pombe*, *Ca*Tor1 regulates the level of Brg1 indirectly via the Hog1 MAP kinase pathway (122). A study of fluconazole-sensitive mutants in *C. neoformans* also suggests that TORC2 signaling is conserved and may play an important role in recurrent infections of *C. neoformans* (123). Thus, for example, similar to equivalent mutations in *S. cerevisiae*, the deletion of *C. neoformans* Sin1 or Ypk1 resulted in hypersensitivity to inhibition of sphingolipid synthesis, and the mutant cells contained low levels of complex sphingolipids (123).

Rapamycin also induced growth inhibition in filamentous fungi, including the human pathogens *Aspergillus fumigatus* (124) and *Aspergillus nidulans* (125). The single gene encoding the TOR kinase in *A. fumigatus* (*Af*Tor1) is essential for growth. However, the use of a conditionally repressible version of *Af*Tor1 in combination with proteomic analysis identified potential protein targets of *Af*Tor1 that are involved in cell cycle regulation, nutrient sensing, and stress response (124). *Af*Tor1 also regulates siderophore biosynthesis and the adaptation to iron starvation, a response that is critical for the virulence of *A. fumigatus* (124). TOR signaling may play an essential role in *Aspergillus* growth via its function as part of TORC2. Thus, for example, the Sin1 homologue in *Aspergillus niger*, RmsA, is required for hyphal elongation and branching (126) and for viability and stress responses (127), reminiscent of the roles identified for TORC2 in cell polarity and stress response in yeasts.

Conservation in the architecture of TOR signaling and the response to rapamycin also exists in phytopathogenic fungi. Rapamycin inhibits the growth and virulence of phytopathogenic fungi, including several species of *Fusarium* (128, 129). Moreover, it was demonstrated that TOR participates in nitrogen metabolite repression (e.g., in *Fusarium fujikuroi* [128]). In addition, the Tap42-type 2A phosphatase module (129) and Sch9 homologues (130) were identified as downstream targets of TOR signaling in *Fusarium graminearum*. Still, better understanding of the response of pathogenic and filamentous fungi to rapamycin or other TOR inhibitors awaits further identification of TOR components, especially the characterization of the distinct cellular roles of TORC1 and TORC2 and the identification of additional components of these pathways.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Since its early discovery as the target for action of rapamycin, TOR has emerged as a central signaling pathway that regulates many different aspects of growth in response to environmental changes, in particular nutritional changes. Regulation of TORC1 in response to nitrogen availability and/or amino acids is the most well-established nutritional response for the TOR pathway. TORC2 may also respond to nutritional changes, but the mechanisms involved are far less well understood. How TOR signaling mediates response to stress conditions, other than nutritional stress, also remains to be determined. The finding that TOR complexes often act as part of feedback loops has suggested that TOR may function primarily as a mediator of cellular homeostasis (17).

The identification of the two TOR complexes marked a breakthrough in our understanding of TOR signaling pathways and rapamycin response. TORC1 and TORC2 are structurally and functionally distinct, and the search for specific TORC1 and TORC2 downstream and upstream effectors is still the focus of many studies. More recent studies suggest that understanding the regulatory links between TORC1 and TORC2 is also critical for understanding the role of TOR in growth control. *Sp*TORC1 and *Sp*TORC2 oppositely regulate several responses to nitrogen starvation including sexual development and transcriptional reprogramming (131). mTORC1 negatively regulates mTORC2 through phosphorylation of several mTORC2-specific components, while mTORC1 and mTORC2 also converge to control certain aspects of metabolism (86). Future studies are expected to unravel the links between TORC1 and TORC2 in more detail.

Finally, *S. cerevisiae* has played a key role in deciphering the mode of action of rapamycin, a drug that has already proved most valuable for the treatment of several human pathologies. The powerful tools of yeast genetics are expected to contribute to further understanding of the mode of action of rapamycin and second-generation ATP-competitive TOR inhibitors. TORC2-specific inhibitors have been much sought after. Whether yeast will also contribute to finding such valuable research tools and possible drugs is an exciting option that at present remains an open question.

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Fungal Genetics and Genomics as Models for Biology

V

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Fungal Cell Cycle: A Unicellular versus Multicellular Comparison

26

Ilkay Dörter and Michelle Momany

DNA is the master instruction set, encoding everything that makes cells work. So it is no surprise that faithful duplication of DNA and proper distribution of new copies to daughter cells is highly choreographed and tightly controlled. The appearance of nuclei changes in predictable patterns during the cell cycle, passing through the morphologically uneventful interphase into the dramatic rearrangement of mitosis. The core cell cycle machinery is highly conserved among eukaryotes. Many of the mechanisms that control the cell cycle were worked out in fungal cells, taking advantage of their powerful genetics and rapid duplication times.

The critical insight made by researchers working with fungi in the late 1970s and early 1980s was that distinct cell morphologies could serve as landmarks indicating cell cycle stage. This insight combined with the elegant use of conditional mutants allowed dissection of the control of cell cycle progression, the dependency relationships, and checkpoints that ensure fidelity (1). Hartwell and coworkers used temperature-sensitive mutants arrested in the emergence and enlargement of the bud, the cell division cycle (*cdc*) mutants, to follow the state of the single nucleus of *Saccharomyces cerevisiae* (2). Similarly, Nurse and coworkers used conditional mutants arrested at specific cell sizes, also called cell division cycle (*cdc*) mutants, to follow the nucleus of the fission yeast *Schizosaccharomyces pombe* (3, 4). Morris and coworkers used conditional mutants with abnormal nuclear number and morphology, the blocked in mitosis (*bim*) and never in mitosis (*nim*) mutants, to follow the multiple nuclei of the filamentous fungus *Aspergillus nidulans* (5, 6). In this review we will focus on an overview of the cell cycle in the unicellular budding yeast *S. cerevisiae* and compare and contrast it with the cell cycle of the multicellular filamentous fungus *A. nidulans*, pointing out orthologous gene products where they are known (Table 1). For both fungi, we will consider the cell cycle only during vegetative growth because this is where most studies have been done. It should be noted, however, that in reproductive growth (sporulation) basic cell cycle modules are undoubtedly regulated in different ways.

Though much of the basic cell cycle machinery is evolutionarily conserved, the fact that *S. cerevisiae* is uninucleate and unicellular while *A. nidulans* is multinucleate and multicellular leads to significant differences in cell cycle regulation. In *S. cerevisiae* the single nucleus moves to the neck for division, and septum formation and cytokinesis occur as mitosis ends (7). This tight coupling of mitosis, septation, and cytokinesis means that budding yeast cells always have a single nucleus. In contrast, septation does not follow every mitosis in the filamentous fungus *A. nidulans*, and cytokinesis results in partitioning of cells rather than separation (Fig. 1). Typically, cell cycle studies in *S. cerevisiae* begin with vegetatively budding cells. In contrast, cell cycle studies in *A. nidulans* begin with dormant, asexual spores (conidia). The first two or three rounds of mitosis take place in the initial cellular compartment formed when the conidium breaks dormancy and establishes polarity. After the cell passes a critical size threshold, the next mitosis (typically the third or fourth division, depending on conditions) triggers formation of the first septum dividing the hypha asymmetrically into two compartments (8). Subsequent mitotic divisions are also followed by septation, with the tip cell remaining mitotically active and subapical cells arresting in interphase until branch formation (9).

Many of the cellular events underlying these morphological shifts in *S. cerevisiae* and *A. nidulans* have been described and are themselves landmarks; that is,

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Table 1 Cell cycle proteins discussed in text[a]

Protein function	*S. cerevisiae*	*A. nidulans*	Function
Cell cycle machinery	Cdc28	NimX	Mitotic cyclin-dependent kinase (CDK)
	Clb1		B-type cyclin required for transition from G2 to M phase
	Clb2	NimE	B-type cyclin required for transition from G2 to M phase
	Clb3-4		B-type cyclins needed for formation and elongation of mitotic spindle
	Clb5-6		B-type cyclins that induce replication in S phase
	Cln1-3		G1 cyclins needed to pass START and enter S phase
	Far1		Sc: inhibitor of Cdc28-Cln complex
	Kin3	NimA	Kinase Sc: possible role in DNA damage response An: essential for entry into M phase
	Mih1	NimT	Phosphatase that modifies CDK
	Sic1		Sc: Repressor of B-type cyclins, prevents premature entry into S phase
	Swe1	AnkA	Kinase that phosphorylates CDK and inhibits entry into M phase
FEAR and MEN/SIN networks	Cdc5	PlkA	Polo-like kinase Sc: role in FEAR and MEN pathways An: needed for initiation and progression through M phase
	Cdc14		Phosphatase Sc: needed in MEN
	Cdc15	SepH	Kinase Sc: needed in MEN, promotes mitotic exit An: involved in septation
	Dbf2	SidB	Kinase Sc: MEN component and SPOC component An: SIN component, required for septation and conidiation
	Esp1	BimB	Peptidase Sc: promotes sister chromatid separation An: required for progression through M phase
	Fob1		Negative regulator of FEAR network
	Hct1		Needed for MEN; activator of APC/C complex
	Mob1	MobA	Sc: component of MEN, required for Cdc15-dependent phosphorylation of Dbf2 An: SIN component required for septation and conidiation
	Net1		Inhibitor of Cdc14, keeps Cdc14 in nucleolus
	Slk 19		Promotes Cdc14 release from nucleolus
	Spo12		Promotes Cdc14 release from nucleolus by antagonizing Fob1 function
	Swi5		Transcription factor that triggers the transcription of the CDK inhibitor Sic1
DNA damage and replication checkpoint	Chk1	ChkA	DNA damage response kinase
	Ddc2	UvsD	DNA damage checkpoint protein Sc: binds RPA and ATRIP
	Mec1	UvsB	ATR ortholog Sc: DNA damage sensor for processed DSBs An: DNA damage sensor and inhibits septation in hyphae with DNA damage
	Mre11	MreA	DNA repair protein Sc: MRX subunit, part of the intra-S phase checkpoint
	Pds1		Phosphorylated Pds1 blocks sister chromatid separation
	Rad50	SldI	DNA damage response protein Sc: MRX subunit
	Rad51	UvsC	DNA damage response protein Sc: replaces RPA in DSB repair An: mutants sensitive to DSB
	Rad53	ChkC	DNA damage response protein
	Tel1	AtmA	

(Continued)

Table 1 *(Continued)*

Protein function	*S. cerevisiae*	*A. nidulans*	Function
			ATM ortholog
			Sc: DNA damage sensor for unprocessed DSBs
			An: regulates DNA damage checkpoint, required for polarized growth
	Xrs2	ScaA	DNA repair protein
			Sc: MRX subunit
Spindle assembly and spindle position checkpoints	Bfa1		SPOC, negative regulator of Tem1, also component of MEN
	Bub1	SldA	SAC protein kinase prevents progression into anaphase in presence of spindle damage
	Bub2	BubA	Sc: SPOC, forms complex with Bfa1, also component of MEN
			An: putative SIN component
	Bub3	SldB	SAC protein kinase prevents progression into anaphase in presence of unattached kinetochores
	Cdc20	Cdc20	SAC; activator of APC/C complex
	Ipl1	Aurora	SAC Aurora kinase
	Kin4	KfsA	SPOC kinase
			An: present at septa but not SPBs
	Knl1		SAC KMN complex member
	Lte1		SPOC, positive regulator of Tem1, also component of MEN
	Mad1	Mad1	SAC
			Sc: forms complex with Mad2 and inhibits APC/C
			An: localizes to NPC
	Mad2	Mad2	SAC
			Sc: forms complex with Mad2 and inhibits APC/C
			An: localizes to NPC
	Mps1	Mps1	SAC kinase
			Sc: required for SPB duplication
			An: essential protein kinase
	Ndc80	Ndc80	SAC KMN complex member
			Sc: acts as a scaffold for Mad1/Mad2 complex
	Tem1	SpgA	SPOC, GTPase
			Sc: termination of M phase, also component of MEN
			An: roles in mitotic exit and SIN
Morphogenesis checkpoint	Cdc3	AspB	Septin
			Sc: found in septal ring at mother-bud neck
			An: found at forming septa and conidia, but not shown to be involved in checkpoint
	Cdc10	AspD	Septin
			Sc: found in septal ring at mother-bud neck
			An: found at forming septa and conidia, but not shown to be involved in checkpoint
	Cdc11	AspA	Septin
			Sc: found in septal ring at mother-bud neck
			An: found at forming septa and conidia, but not shown to be involved in checkpoint
	Cdc12	AspC	Septin
			Sc: found in septal ring at mother-bud neck
			An: found at forming septa and conidia, but not shown to be involved in checkpoint
	Elm1		Kinase, recruits Hsl1 to septin ring at mother-bud neck
	Hsl1		Kinase, recruits Hsl7 to septin ring at mother-bud neck
	Hsl7		*N*-Methyltransferase, required for Swe1 recruitment to mother-bud neck

[a]Though orthologs have been predicted based on amino acid conservation, only orthologs with experimental data are included here. Functions are based on annotations in SGD (http://www.yeastgenome.org/) and AspGD (http://www.aspgd.org/) and on references cited in the text. Sc: ortholog in *S. cerevisiae*; An: ortholog in *A. nidulans*. Other abbreviations: DSB, double-strand break; MEN, mitotic exit network; SIN, septation initiation network; SPOC, spindle position checkpoint.

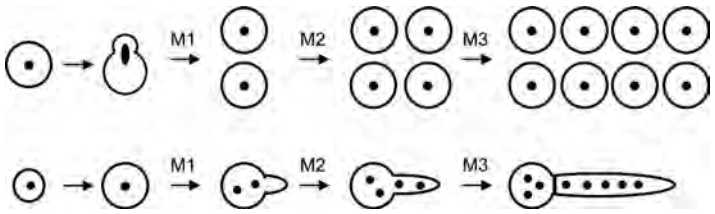


Figure 1 Mitosis, septation, and cytokinesis in *Saccharomyces cerevisiae* and *Aspergillus nidulans*. Three rounds of mitosis in *S. cerevisiae* beginning from vegetatively growing yeast cell (top) compared to *A. nidulans* beginning from dormant conidium (bottom). Black dots are nuclei. M1, M2, and M3 denote the indicated mitotic divisions.

they always occur with the same stage of the cell cycle and thus can be used to anchor and order observations.

S. CEREVISIAE LANDMARKS

The unicellular yeast *S. cerevisiae* propagates by budding, and the cell must grow to a critical size before it initiates a new cycle. Attaining this critical size in the G1-phase (gap 1) of the cycle allows the cell to pass START, which is defined as the point at which the cell irreversibly commits to the mitotic cell cycle (10). The size threshold at START prevents cell size decrease through premature cell cycle progression (11). Though the mechanisms are not clear, START appears to involve some of the same cyclin-dependent kinases (CDKs) and cyclins that drive the cell cycle (see below) (12).

After passing START, the cell enters the S phase (synthesis phase), where DNA and the spindle pole body (SPB) are duplicated (Fig. 2). The SPBs are microtubule organizing centers, functionally equivalent to centrosomes of higher organisms (7). However, unlike in higher eukaryotes, the nuclear envelope does not break down in *S. cerevisiae*, so the SPBs are embedded in the nuclear envelope. During S phase the SPBs separate, forming short microtubule spindles between them. The SPBs continue to separate, eventually reaching the opposite poles of the nucleus, where they anchor the medial spindles thought to function in metaphase (13).

The presence of short spindles during S phase is unusual. In most organisms, spindle formation is one of the defining features of M phase (14). The presence of short spindles during S phase has led to disagreement about whether *S. cerevisiae* has a true G2 phase; consequently, many *S. cerevisiae* researchers use the designation "G2/M." However, the evolutionary conservation of cell cycle processes and careful measurements suggesting that DNA replication finishes before medial spindle assembly has led others to designate a separate G2 phase (7). To make comparisons between *S. cerevisiae* and *A. nidulans* easier, we will use the convention of separate G2 and M phases in this review.

Late in S phase, the actin cytoskeleton polarizes toward a region on the cell surface and directs secretory vesicles to the site of future polarity establishment (15). This leads to the formation of a bud, certainly the easiest S phase landmark to spot in *S. cerevisiae* (Fig. 2).

The G2-phase (gap 2) follows the successful completion of S phase and is characterized by rapid cell growth and protein synthesis, which prepare the cell for mitosis. The M phase (mitotic phase) is subdivided into prophase, metaphase, anaphase, and telophase (Fig. 2). During the closed mitosis of *S. cerevisiae*, regulated active transport through the nuclear pore complexes still occurs (14). During prophase the chromatin condenses and the microtubules of the mitotic spindle attach to the kinetochores. In contrast to higher eukaryotes, yeast chromosomes do not line up at the midzone of the spindle to form a metaphase plate (16). The absence of a metaphase plate can make metaphase difficult to detect, but it is generally defined as the point when all chromosomes show bipolar attachment to the spindle microtubules (13). During anaphase sister chromatids separate and travel to opposite poles and are equally distributed between the mother and daughter cells (17). In telophase chromosomes decondense and cytokinesis rapidly follows, separating mother and daughter cells by contraction of the actomyosin ring and synthesis of a new cell wall between the cell bodies (18).

A. NIDULANS LANDMARKS

While a START commitment point has not been characterized in *A. nidulans*, it seems likely that an analogous size threshold is present, because G1-arrested dormant conidia (19) all expand to a similar size before germ tube emergence (M. Momany, personal observations). Any such size threshold in conidia would be

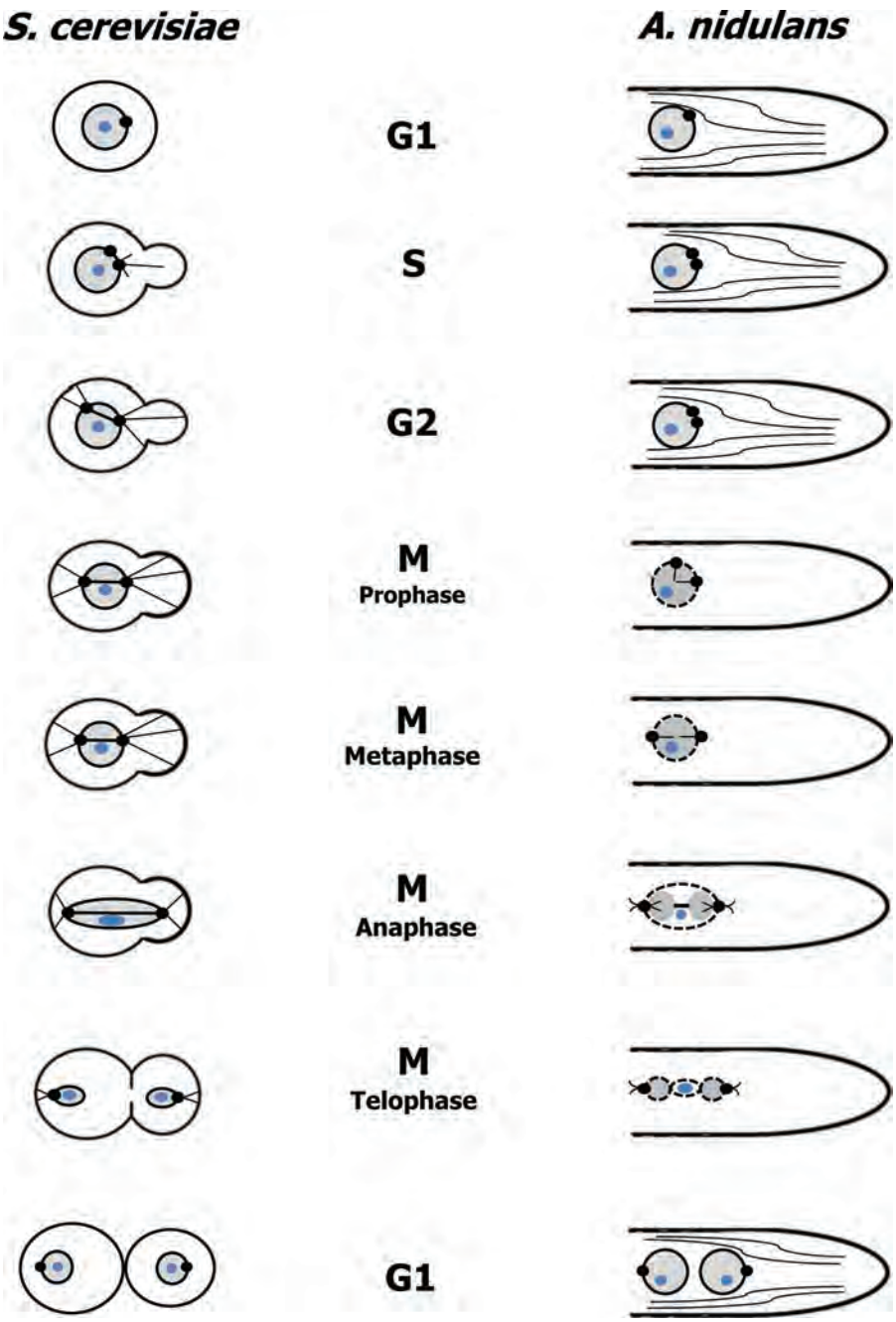


Figure 2 Cell cycle landmarks in *Saccharomyces cerevisiae* and *Aspergillus nidulans*. Major morphological landmarks for each cell cycle stage are shown for *S. cerevisiae* (left) and *A. nidulans* (right). Gray circle, nucleus; solid border, intact nuclear envelope; broken-line border, partial nuclear pore complex disassembly; small black dots, spindle pole bodies; blue dots, nucleolus; lines, microtubules. (Data from references 117 and 120.)

used only once before emergence of the first germ tube, as opposed to START in yeast, which occurs in every cell cycle. It is possible that other commitment points gate entry into subsequent cell cycles in *A. nidulans*. Certainly, a critical size threshold must be crossed in hyphae before septum formation (cytokinesis) can be triggered (20). In S phase (Fig. 2), the nucleus undergoes replication and the spindle pole body is duplicated

(14, 21). The two SPBs do not separate until the cell passes the G2/M boundary (22). As the cell enters mitosis the cytoplasmic microtubules depolymerize (23) and the nuclear pore complexes (NPCs) partially disassemble (14). The partial disassembly of the NPCs allows tubulin molecules to enter and form spindle microtubules originating from the nuclear face of the SPBs (22, 24). In prophase the SPBs move apart (25). Similar to *S. cerevisiae*, the bipolar attachment of chromosomes to the spindle does not trigger the formation of a distinct metaphase plate. During anaphase as the sister chromatids begin to move toward the poles, microtubules between the two SPBs elongate, astral microtubules form on the cytoplasmic face of the SPBs (26), and the segregating DNA is separated from the nucleolus. The nucleolus is excluded to the cytoplasm within telophase, and nucleolar proteins are reimported into the daughter nuclei during G1 (27).

CDKs AND CYCLINS DRIVE CELL CYCLE PROGRESSION

The cell cycle is driven by the interaction of the CDKs with cyclins (7). Cyclins, so named because of their periodic, cyclical expression (28), bind CDKs and regulate their phosphorylation of target proteins. The CDKs and cyclins are themselves regulated via phosphorylation. One of the best-studied CDK/cyclin pairs is CDK1 and cyclin B, which controls entry into mitosis. Many of the details of CDK/cyclin interaction were worked out in *S. pombe*, so the S. *pombe* naming conventions are used. CDK1/cyclin B is phosphorylated by the Wee1 kinase, inhibiting entry into mitosis, and is dephosphorylated by the Cdc25 phosphatase, allowing mitosis to proceed (Fig. 3). As might be expected for such critical control pathways, CDK-cyclin complexes are also regulated by other mechanisms including binding by inhibitors, transcription, and proteolytic degradation. Targeted proteolysis of cell cycle regulators by the ubiquitin proteasome system, including cyclins, helps make the cell cycle irreversible and unidirectional, which imposes a further control mechanism. The degradation takes place at three different time points: during the transition between G1 and S phase, before anaphase, and at mitotic exit (29).

S. cerevisiae Cell Cycle

In *S. cerevisiae* the CDK regulating cell cycle event is Cdc28 (7). Yeast cyclins are divided into two classes: the G1 cyclins Cln1 to Cln3, which predominantly regulate events at the end of G1 and are essential to pass START (30), and the B-type cyclins Clb1 to Clb6, which are expressed between START and mitotic exit (31). CDK-cyclin complexes are themselves regulated by phosphorylation and dephosphorylation (Fig. 3).

G1 phase

Cln3 is expressed early in G1 together with the Cdc28-Cln inhibitor Far1 (32). As G1 progresses levels of Far1 fall below the threshold needed to inhibit the Cdc28-Cln3 complex (33). This allows Cdc28-Cln3 to stimulate the transcription of G1 cyclins including Cln1 and Cln2, pass START, and trigger entry into S-phase (34).

S phase

The Cdc28-Cln1 and Cdc28-Cln2 complexes initiate DNA replication and the events that lead to bud emergence and SPB duplication, two main S phase landmarks (35). In addition, the Cdc28-Cln2 complex stimulates the degradation of Sic1, a repressor of B-type cyclins (36, 37). This enables the activation of the Cdc28-Clb5 and -Clb6 complexes, which induce DNA replication and ensure entry into S phase (38, 39). Cdc28-Clns trigger their own degradation via autophosphorylation (40). S phase ends when DNA synthesis is correctly completed (see "DNA Damage and Replication Checkpoints", below).

G2 phase

As described in the discussion of *S. cerevisiae* landmarks above, the presence of short spindles during S phase in *S. cerevisiae* has led to disagreement about precisely when M phase starts and the existence of G2 phase.

M phase

To enter into M phase the Cdc28-Clb2 (CDK-cyclin B) complex must be activated (Fig. 3). In G2 the Cdc28-Clb2 complex is inhibited by phosphorylation by the Swe1 kinase (41). The inhibitory phosphate is removed by the phosphatase Mih1. The degradation of Swe1, mediated by the morphogenesis checkpoint (see below), generates a positive feedback loop that is responsible for a rapid increase of active Cdc28-Clb complexes. This ensures the entry into M phase and subsequent bud growth (42, 43). In conjunction with Cdc28, the mitotic cyclins Clb1 to Clb4 control the initiation and the progression through the cell cycle. Cdc28-Clb3 and Cdc28-Clb4 trigger the formation and elongation of the mitotic spindle, whereas Cdc28-Clb1 and Cdc28-Clb2 activate the anaphase promoting complex APC/C.

The APC/C degrades G1 cyclins, B-type cyclins, and securin, whose loss initiates the separation of sister chromatids (44). The expression of the Cdc28-Clb

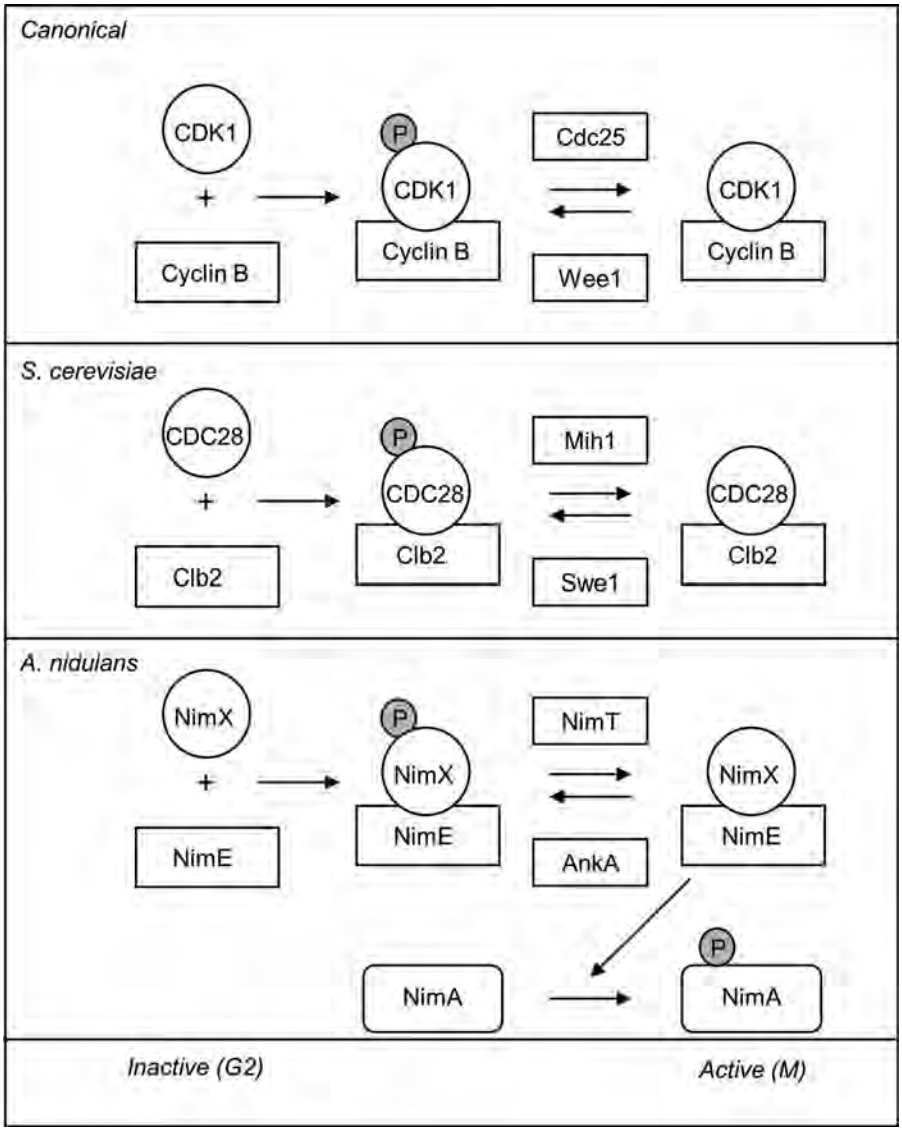


Figure 3 Cyclin-dependent kinase (CDK)/cyclin B regulation. Comparison of CDK and cyclin activity at G2/M phases in *Schizosaccharomyces pombe* (canonical), *Saccharomyces cerevisiae*, and *Aspergillus nidulans*. (Data from reference 52.)

inhibitor Sic1 and activation of the phosphatase Cdc14 is also required for mitotic exit. Sic1 represses B-type cyclins, and Cdc14 dephosphorylates substrates of Cdc28, which reinitiates the G1-phase (45, 46).

The activity of Cdc14 is controlled by the inhibitor Net1, which keeps Cdc14 anchored in the nucleolus and inactive throughout the G1, S, G2, and early M phases (47). Two pathways control the release of Cdc14: the FEAR (Cdc fourteen early anaphase release) network and MEN (mitotic exit network) (Fig. 4). The FEAR network releases Cdc14 from the nucleolus during early anaphase, and the MEN maintains Cdc14 in the released state during late anaphase and telophase. The MEN includes several components that are also part of the spindle assembly and position checkpoints. These are discussed in more detail in the checkpoint section of this review.

The FEAR network includes the separase Esp1, the kinetochore protein Slk19, the nucleolar proteins Spo12 and Fob1, and the polo kinase Cdc5 (48). The protease Esp1 is responsible for the cleavage of cohesins at the onset of anaphase. Slk19 is also a target of this protease, but its role in the FEAR network is still unclear. Cdc5 promotes the activity of the spindle position

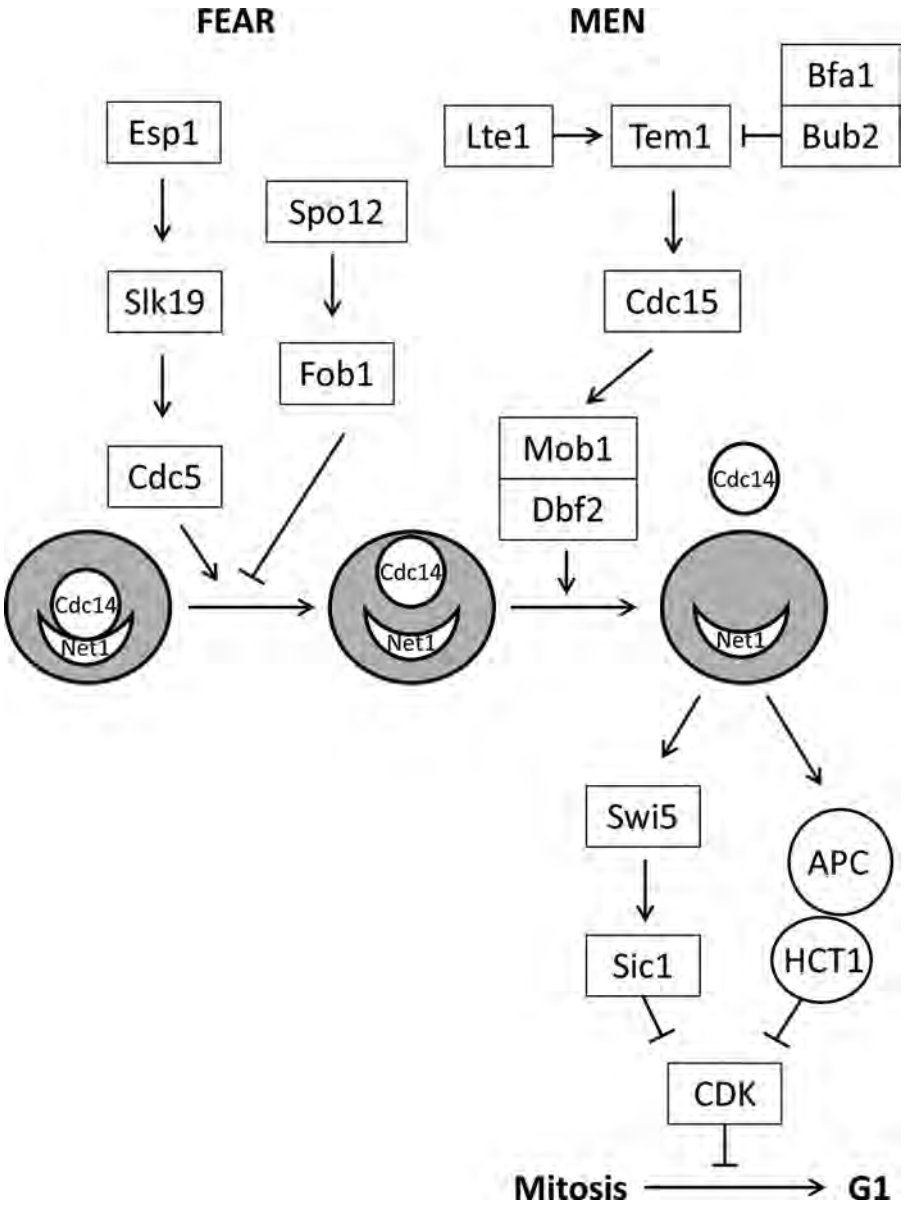


Figure 4 Model of the Cdc fourteen early anaphase release (FEAR) network and mitotic exit network (MEN) at the end of mitosis. In early anaphase in *Saccharomyces cerevisiae* the FEAR network promotes the release of Cdc14 from the nucleolus. In late anaphase the MEN releases Cdc14 from the nucleus into the cytoplasm, where it activates both Swi5 and Hct1. Swi5 triggers the transcription of the cyclin-dependent kinase (CDK) inhibitor Sic1. Hct1 binds the anaphase-promoting complex (APC), and this complex marks Cdk1 for degradation. These events cause the cell to exit mitosis and enter into a new cell division cycle. Gray circles represent the nucleus. (Modified from reference 121 with permission.)

checkpoint protein Dbf2 and probably activates Bub2 and Bfa1 (47). Cdc5 is essential for the release of Cdc14 from the nucleolus and for its maintenance in the released state (49).

The MEN (Fig. 4) consists of several proteins that are part of the spindle assembly checkpoint (SAC) including the GTPase Tem1, its negative regulators Bub2 and Bfa1, and its positive regulator the GDP-GTP exchange factor Lte1. After interacting with Lte1, Tem1 activates Cdc15. The protein kinase Cdc15 then activates the kinase Dbf2 through phosphorylation. Phosphorylated Dbf2 activates proteins involved in septum

formation and cytokinesis (50). Tem1 localizes to the SPB and migrates into the daughter cell during anaphase, whereas Tem1's exchange factor Lte1 localizes to the bud. Tem1 and Lte1 are in the same cellular compartment when the nucleus enters the daughter cell. This ensures that mitotic exit occurs only when the genetic material is distributed between mother and daughter cells (51).

A. nidulans Cell Cycle

In *A. nidulans* NimX is the CDK equivalent of the *S. cerevisiae* Cdc28 (52) (Fig. 3). The only characterized mitotic cyclin is NimE, a homolog of cyclin B, though cyclins involved in development have been investigated (53), and putative cyclin orthologs have been identified based on sequence conservation (54). In addition to the CDK NimX, *A. nidulans* requires a second kinase, NimA, for entry into mitosis. The NimX and the NimA kinases are responsible for the initiation of mitosis in *A. nidulans*, and their inactivation is required for progression through mitosis (55, 56). Interestingly, the *S. cerevisiae* homolog of NimA, Kin3, is not essential and appears to play a role in DNA damage response.

G1 phase

A. nidulans orthologs of the G1 cyclins have been computationally predicted, but they have not been characterized (54). The Ca^{2+}/calmodulin-regulated kinases CMBK and CMKC have both been reported to have roles in progression through G1/S, though how they interact with the other cell cycle machinery is not yet clear (57).

S phase

The CDK cyclin machinery that drives *A. nidulans* entry into and exit from S phase is not well understood, though more is known about the DNA replication checkpoints that allow exit from S phase when DNA synthesis is correctly completed (see checkpoints below).

G2 phase

During interphase NimX is associated with NimE (cyclin B) and phosphorylated by AnkA, the *A. nidulans* homolog of the *S. cerevisiae* phosphatase Swe1 (52, 58, 59). NimX is inhibited (G2 arrest) by AnkA phosphorylation and activated by NimT dephosphorylation (60). However, activation of NimX alone is not sufficient to trigger mitotic entry. A temperature-sensitive mutant version of the NimA kinase (*nimA5*) causes a late G2 arrest at restrictive temperature (61), while the NimX/NimE complex is fully active at the *nimA5* arrest point (62). This demonstrates that NimA is required for the G2/M transition by a mechanism independent from that of NimX. NimX and NimE are excluded from nuclei in G2-arrested *nimA* mutants (59), suggesting that NimA regulates mitotic entry by affecting the localization of NimX/NimE. NimA is located in the cytoplasm throughout G2 (24).

M phase

To exit from G2 and enter into M phase, the NimX-NimE (CDK-cyclinB) complex must be activated (Fig. 3). In G2 NimX is inhibited by phosphorylation by the AnkA kinase. To move into M phase the NimX CDK must be activated by the NimT phosphatase (60). Once activated, the NimX-NimE (CDK-cyclin B) complex phosphorylates and activates the NimA kinase. Subsequently, NimA moves into the nucleus, locates at the spindle poles during mitosis, and is degraded at mitotic exit (63, 64).

Mitotic exit in *S. cerevisiae* is mediated by the MEN. The equivalent network in the fission yeast *S. pombe*, known as the septation initiation network, has been extensively studied. As noted above, in *S. cerevisiae* mitotic exit and cytokinesis are tightly coupled, but they are temporally separated in *A. nidulans*. Mitotic exit in *A. nidulans* is more similar to the *S. pombe* septation initiation network than to the *S. cerevisiae* MEN (65). Both the polo kinase Plo1 and the cyclin-dependent kinase Cdc2/Cdc13 activate the septation initiation network in *S. pombe* and coordinate it with mitotic events (66). Once activated, the upstream GTPase Spg1 activates the downstream protein kinase cascade Cdc7 to Sid1 to Sid2 (67). Ultimately, the Sid2/Mob1 complex is relocated from the spindle poles to the contractile actin ring, where it is required for ring contraction and septum deposition (i.e., cytokinesis) (68).

In *A. nidulans* repeated mitotic divisions without cell separation lead to the formation of a mycelium of multinucleate cells that are separated by multiple septa. Strains carrying a temperature-sensitive mutation in the Ser/Thr protein kinase SepH resulted in a viable strain that failed to septate at restrictive temperature (69). Further studies suggested that SepH is required for the construction of the actin ring (70). It is likely that SepH, which catalyzes the initial event of septum formation, collaborates with other proteins such as SidB and MobA (70, 71). In *S. pombe* Mob1, the ortholog of MobA, is associated with Sid2, the ortholog of SidB. Mutant versions of SepH, SidB, or MobA do not impair hyphal growth or colony formation in *A. nidulans*, whereas their respective orthologs Cdc7, Sid2, or Mob1 are essential for cytokinesis in *S. pombe*. This shows that cytokinesis is essential in the uninucleate

fission yeast but not in the vegetative growth of the multinucleate, filamentous fungus *A. nidulans* (71).

CHECKPOINTS GUARD CELL CYCLE TRANSITIONS

As described above, there are several CDK-cyclin-dependent mechanisms that establish the correct order of cell cycle events including size control at START, phase-specific gene expression, posttranslational modification (especially phosphorylation and dephosphorylation), and targeted proteolysis. In addition, critical cell cycle events are monitored by a series of checkpoints that halt progression to the next phase until key events are complete and error free (72). A checkpoint generally consists of sensors that monitor the completion of a cell cycle event, transducers which forward information from the sensor, and effectors that halt cell cycle progression (73) (Fig. 5). Checkpoints are usually identified through the use of toxic agents that cause major damage and thus impose an exaggerated cell cycle delay while damage is repaired, but surveillance of major cell cycle events by checkpoints occurs under normal (nonstressed) growth conditions too (74, 75). For example, the cell uses many of the same proteins to perform routine DNA repair and to trigger the DNA damage checkpoint (76).

DNA Damage and Replication Checkpoints

The evolutionarily conserved DNA damage checkpoint ensures that DNA is correctly and completely replicated before mitosis is triggered. This checkpoint is one branch of the DNA damage response pathway (or network) that also includes branches that trigger DNA repair, damage-induced transcription, and in multicellular organisms, apoptosis (76). The DNA damage checkpoint is triggered by double-strand breaks (DSBs) or replication fork stalls. Depending on when the checkpoint is triggered, cell cycle progression can be delayed or halted at the G1/S or G2/M boundaries by downregulating the appropriate CDK, or the checkpoint can slow down replication by preventing more origins from firing (sometimes called the "intra-S" or "slowed-S" checkpoint) (77–79). The G2/M block in the DNA damage checkpoint is covered below with an emphasis on checkpoint-specific components rather than replication and repair machinery that also play roles in the checkpoint.

S. cerevisiae

In *S. cerevisiae* the sensors for DNA damage can include repair and replication complex proteins (which can also be effectors) along with phosphatidylinositol-3-OH-like kinases (79). The sensor for processed DSBs and replication fork stalls is the phosphatidylinositol-3-OH-like kinase Mec1 (ATR in mammals), and for unprocessed DSBs the sensor is Tel1 (ATM in mammals) (80, 81). Both of these DNA damage sensors and their interacting proteins are physically associated with the DNA repair machinery and replication forks (Fig. 6).

Before DNA is replicated in S phase, the double helix is opened up by a multiprotein complex. DNA binding proteins, helicases, polymerases, and other specialized proteins are recruited to form the replisome which makes new DNA strands bidirectionally at the fork. Replisomes can stall when they hit obstacles or when nucleotide pools are low. When replisomes stall, helicase and polymerase activities are uncoupled, which produces excess single-stranded DNA (ssDNA). It is this excess ssDNA along with the RPA (replication protein A) that coats it which appears to be the physical trigger for the DNA damage checkpoint. Ddc2, the *S. cerevisiae* ATR interacting protein ortholog, binds to RPA and associates with Mec1, thus targeting it to sites of DNA damage.

To repair DSBs, multiple proteins are recruited to the lesion, including the MRX complex (Mre11, Rad50, Xrs2; called MRN in mammals). The MRX complex in turn recruits the *S. cerevisiae* ATM ortholog Tel1. In the process of repairing DSBs, the DNA repair machinery makes 3′ssDNA tails. The RPA complex coats these ssDNA tails, which leads to recruitment of Mec1 and DNA damage checkpoint activation. RPA is later displaced by Rad51 and Rad52.

Several activators of Mec1 (the ATR kinase) are needed for its full activity, including Rad17-Mec3-Ddc1 (the "9-1-1 complex" in mammals) (81). Activated Mec1 phosphorylates DNA damage response kinases Rad53, Chk1, and Dun1. In *S. cerevisiae* the DNA damage checkpoint ultimately blocks sister chromatid separation by phosphorylation of Pds1. In most other organisms, including mammals and *S. pombe*, the DNA damage checkpoint acts by phosphorylation of the CDK1 and a block at the G2/M boundary, often through inhibition of the Cdc25 phosphatase.

A. nidulans

The *A. nidulans* DNA damage checkpoint appears to have the same basic structure as that of *S. cerevisiae*. *A. nidulans* has an ATR kinase ortholog, UvsB, and an ATM kinase ortholog, AtmA. Based on sensitivity of deletion mutants to genotoxic agents, the *A. nidulans* ATM appears to be mainly involved in repair of DSBs,

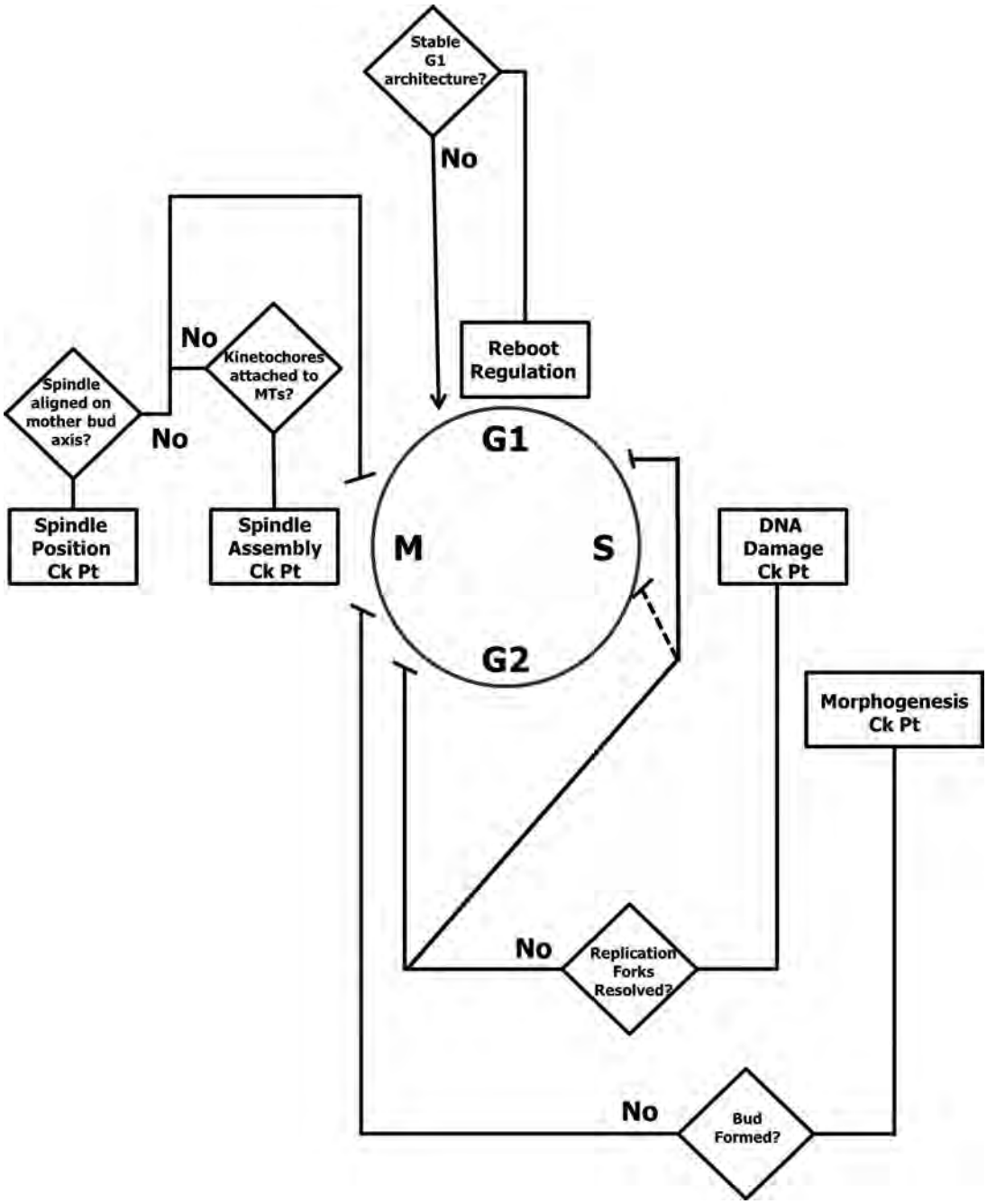


Figure 5 Cell cycle checkpoints in *Saccharomyces cerevisiae* and *Aspergillus nidulans*. Sensors (depicted as rectangles) monitor the completion of specific cell cycle events (depicted as diamonds) and forward the information to effectors that halt or reboot the cell cycle. G1: Reboot regulation (not reported in *S. cerevisiae*). S phase: DNA damage network includes branches that are responsible for DNA repair and damage-induced transcription. Depending on when the checkpoint is triggered, cell cycle progression can be delayed or halted at G1/S or G2/M boundaries or DNA replication can be slowed (shown as dotted line). S/G2: Morphogenesis checkpoint (not reported in *A. nidulans*). M phase: Spindle assembly checkpoint (SAC) and spindle position checkpoint (SPOC) SAC arrests at metaphase, preventing anaphase. SPOC not reported in *A. nidulans*. (Data from reference 122.)

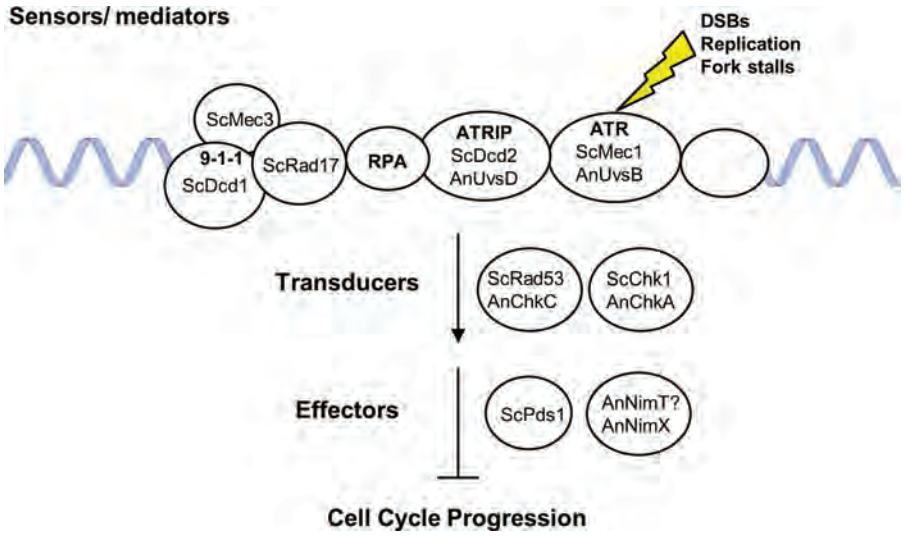


Figure 6 DNA damage checkpoint in *Saccharomyces cerevisiae* and *Aspergillus nidulans*. Sensor proteins monitor DNA damage and transmit the information to transducers and effectors that ultimately modulate the cell cycle. Only proteins discussed in text are shown. DSB, double-strand break; RPA, replication protein A; ATRIP, ATR interacting protein. (Data from reference 80.)

while ATR recognizes both DSB and replication fork stalls (52, 82–86).

Unlike *S. cerevisiae*, but like most other organisms, the DNA damage checkpoint in *A. nidulans* triggers an arrest at the G2/M boundary by inhibitory phosphorylation on NimX, the CDK1/Cdc28 ortholog. It is not yet clear how the Cdc25 phosphatase (NimT) and Wee1 kinase (AnkA) are involved in this process. Also different from *S. cerevisiae*, the *A. nidulans* ATM (AtmA) and ATR (UvsB) appear to be partially redundant because the double ATM/ATR mutant is nonviable (86). In addition to its role in regulating the DNA damage checkpoint, UvsB/ATR inhibits septation in predivisional hyphae that have sustained DNA damage (85, 87, 88). Similarly, in addition to its role in the damage response, AtmA is required for polarized growth (89, 90).

Another possible DNA damage checkpoint in *A. nidulans* includes the nuclear pore protein SonB. The *nimA* G2 arrest can be suppressed by mutations in *sonB*, first identified as a suppressor of *nimA* (91). Certain *sonB* mutants are hypersensitive to DNA damage but have a functional G2 DNA damage checkpoint. These same mutants also show synthetic lethality with mutants in the MRX complex member *scaA*, leading to the suggestion that SonB might play a novel role in the DNA damage response pathway in *A. nidulans* (52, 82).

SACs and Spindle Position Checkpoints

For proper chromosome segregation at anaphase, sister chromatids must be stably attached to opposing spindle microtubules so that they will be pulled in opposite directions. The SAC monitors the attachment of chromosomes to microtubules at kinetochores and prevents the transition from metaphase to anaphase until all sister chromatids are properly attached (92, 93). The transition from metaphase to anaphase is driven by the anaphase-promoting complex/cyclosome (APC/C), a multisubunit E3 ubiquitin ligase, which targets securin, along with other cell cycle proteins, for degradation. The APC/C is activated by binding to Cdc20. The presence of even a single unattached kinetochore triggers the assembly of the mitotic checkpoint complex (MCC), which binds to Cdc20 and prevents it from activating the APC, thereby halting anaphase and cell cycle progression.

S. cerevisiae

The main components of the SAC were discovered in the early 1990s through *S. cerevisiae* mutants that did not arrest in metaphase in the presence of spindle toxins. These include the products of the mitotic arrest deficient (MAD) genes *MAD1*, *MAD2*, and *MAD3* and the budding uninhibited by benzimidazole (BUB) genes *BUB1* and *BUB3*, which are highly conserved in all eukaryotes (94).

Kinetochores are decorated with multiple KMN complexes (Knl1, Mis12, and the Ncd80 complex) that work together like "molecular Velcro" to bind microtubules (93) (Fig. 7). The SAC kinase Mps1 associates with kinetochores in early mitosis, an association that requires the Ncd80 complex and is regulated by the Ilp1 Aurora kinase. Ilp1 detects improper tension between sister chromatids and phosphorylates microtubule binding sites, thereby destabilizing the connection between kinetochores and spindle microtubules. As a consequence, the SAC is activated and SAC components are recruited to the kinetochore to form the MCC (Mad2, Mad3, Bub3, and Cdc20) (92). Mad2 can exist in two conformations: the inactive open form (Mad2-O) and the active closed form (Mad2-C). Through binding to Mad1, Mad2 undergoes a conformational change from inactive (Mad2-O) to active (Mad2-C), forming a stable Mad1-Mad2-C complex (95). This complex is recruited to the unattached kinetochore and serves as a receptor for the recruitment of more inactive Mad2-O protein from the cytoplasm. After binding to the Mad1-Mad2-C receptor, the inactive Mad2-O is converted into an active Mad2-C, which facilitates the binding of Cdc20 (96). The resulting MCC binds the APC/C and inhibits its activity (97, 98). The MCC itself is unstable and must continually be renewed to maintain the SAC block on cell cycle progression in the presence of unattached kinetochores.

APC/C inhibition is released once all sister chromatid kinetochores are properly associated with microtubules. Properly attached kinetochores no longer trigger high levels of Mps1 kinase activity and the resulting Mad1 and Mad2 recruitment. Quick inactivation of the SAC signal also requires dynein, which transports previously activated SAC proteins toward the microtubule poles, and the protein phosphatase 1,

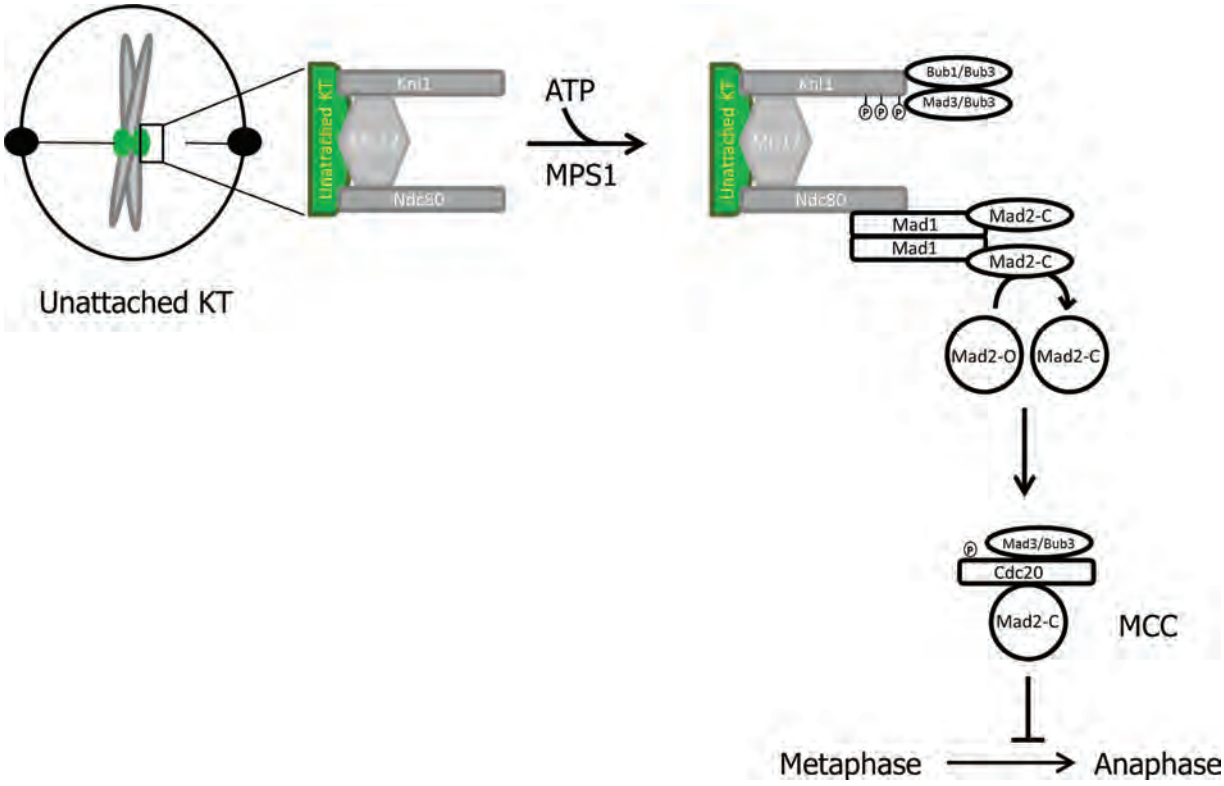


Figure 7 Spindle assembly checkpoint (SAC) in *Saccharomyces cerevisiae*. Unattached kinetochores activate the SAC response by recruiting MPS1, which phosphorylates Knl1. The KMN (Knl1-Mis12-Ndc80) network serves as a scaffold for the recruitment of checkpoint proteins. Binding of Bub1/3 promotes the recruitment of the Mad1/Mad2 core complex. Kinetochore-bound Mad1/Mad2 catalyzes the conformational change of the open Mad2-O to the closed Mad2-C form. Mad2-C interacts with Cdc20 and forms the mitotic checkpoint complex (MCC) consisting of Cdc20, Mad2-C, BubR1, and Bub3. This inhibits the anaphase-promoting complex activator Cdc20 and blocks the metaphase to anaphase transition. KT, kinetochore. (Data from reference 92.)

which dephosphorylates kinetochore substrates necessary for the formation of the MCC (93, 99, 100).

After anaphase is triggered, another checkpoint must be satisfied before *S. cerevisiae* cells are permitted to exit mitosis. The spindle position checkpoint prevents cells with misaligned spindles from exiting mitosis (101) (Fig. 8). Tem1, a RAS-like GTPase that controls the MEN, is the key protein in this process. In response to spindle alignment defects, the GTPase-activating dimer complex Bub2/Bfa1 accelerates GTP-hydrolysis of Tem1, maintaining it in its GDP-bound inactive form. Support is provided by the Ser/Thr kinase Kin4, which protects Bub2/Bfa1 from an inhibitory modification by the polo-like kinase Cdc5 (102). The subcellular localization of these proteins changes during the cell cycle. From G1 to metaphase the inactive Tem1 and Bub2/Bfa1 complex are located at both SPBs. The amount of Tem1 increases at the SPB of the daughter

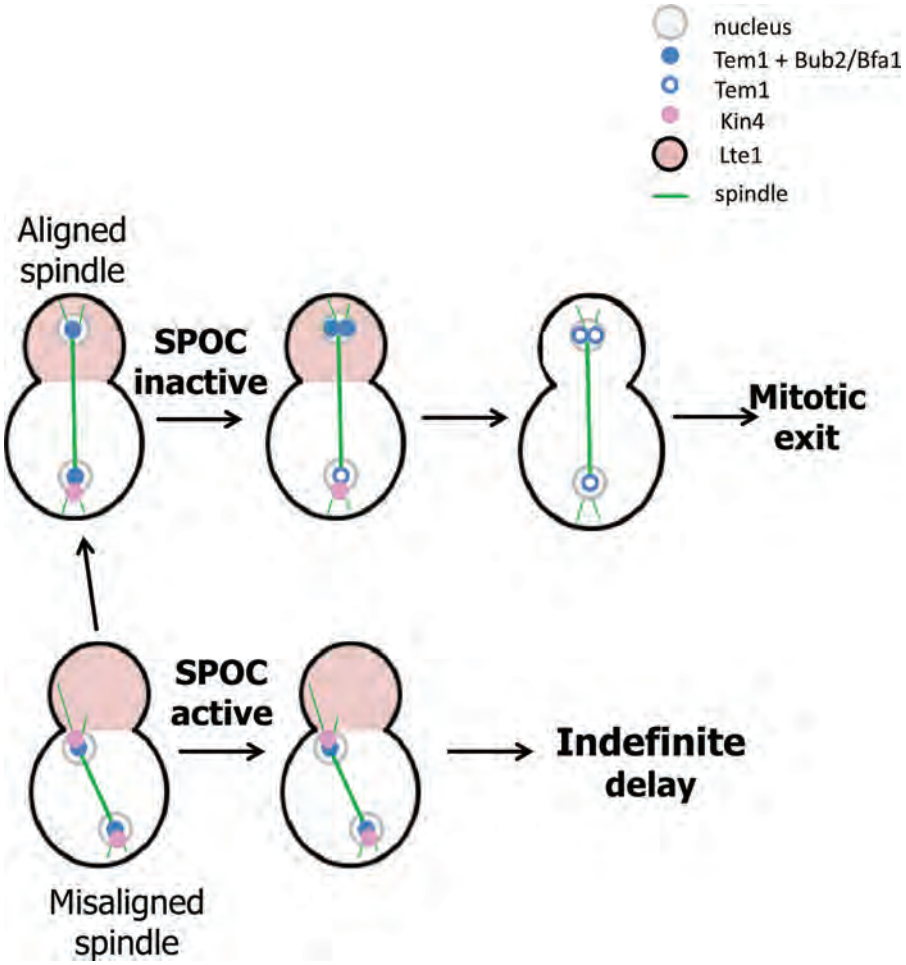


Figure 8 Spindle position checkpoint (SPOC) controls mitotic exit in *Saccharomyces cerevisiae*. When the spindle is misaligned, Bub2/Bfa1 keeps Tem1 in its inactive form. Kin4 protects Bub2/Bfa1 itself from an inhibitory modification. Bub2/Bfa1 disappears from the mother cell once one spindle pole body (SPB) is correctly positioned in the daughter cell. At the same time the amount of Tem1 and Bub2/Bfa1 increases at the SPB of the daughter cell. Lte1, which is located at the cortex of the daughter cell, activates Tem1, and Bub2/Bfa1 is phosphorylated by the polo kinase Cdc5. Bub2/Bfa1 disappears from the daughter cell, whereas Kin4 is removed from the SPB of the mother cell. Simultaneously, Lte1 diffuses into the cytoplasm of the mother and daughter cells. It is possible that all these events contribute equally to the activation of the mitotic exit network. The nucleus is depicted as a gray circle, and the pink highlighted part of the cell shows Lte1 in the cytoplasm. The blue empty circle represents the GTPase Tem1, and the blue filled circle is Tem1 associated with the dimer complex Bub2/Bfa1. (Data from reference 123.)

cell once the correctly positioned spindle passes the bud neck. Here Bub2/Bfa1 is inhibited by the polo kinase Cdc5. Lte1, which is exclusively localized at the cortex of the daughter cell, activates Tem1, which phosphorylates the protein kinase Dbf2 and initiates the MEN (102).

A. nidulans

Much of the SAC machinery is conserved between *A. nidulans* and *S. cerevisiae* (22). Consistent with *A. nidulans* undergoing a semiopen mitosis, in which NPCs partially disassemble, rather than a closed mitosis as in *S. cerevisiae*, *A. nidulans* possesses a layer of additional spatial regulation. In interphase, An-Cdc20 and SldA (the *A. nidulans* homolog of Bub1) are in the cytoplasm, An-Mps1 is at SPBs, An-Mad1 and An-Mad2 are localized to the nuclear periphery, and SldB (the *A. nidulans* homolog of Bub3) is in the nucleoplasm (22, 103). After the partial disassembly of the NPCs at mitosis, An-Mad1 and An-Mad2 enter the nucleus and concentrate at unattached kinetochores. Subsequently, SldA (Bub3) and An-Cdc20 enter the nucleus and accumulate at the SPB/kinetochore complex. An-Cdc20 spreads through the nucleus while SldB leaves it (22). The SAC mitotic arrest in *A. nidulans* has a finite life span and is actively turned off, even when cells cannot make a mitotic spindle (104, 105).

No spindle position checkpoint has been identified in *A. nidulans*. This is not surprising, because in *S. cerevisiae* determination of the cytokinetic division plane precedes mitosis, and the spindle must later be aligned to deposit one set of chromosomes each in mother and daughter (101). Because conidiophore formation in *A. nidulans* requires multiple coordinated budding steps, it is possible that a spindle position checkpoint might be involved in asexual reproduction.

Morphogenesis Checkpoint

In *S. cerevisiae* the morphogenesis checkpoint delays mitosis until the bud is of a suitable size to receive the daughter nucleus. The morphogenesis checkpoint has not been found in other organisms, but it would be surprising if the principle of ensuring proper morphology before mitosis is not eventually found elsewhere.

S. cerevisiae

Yeast cells contain a highly polarized actin cytoskeleton that is responsible for polarized growth and therefore bud formation (106). Perturbation of actin polymerization was shown to delay bud formation but not to uncouple the budding cycle from the cell cycle (107). This suggested the presence of a checkpoint mechanism that monitored bud formation. Mutant strains lacking the protein kinase Swe1 display a functional cell cycle under normal conditions (41) but do not arrest when actin polarization is impaired (108). Under normal conditions, septins, filament-forming cytoskeletal proteins encoded by *CDC3*, *CDC10*, *CDC11*, and *CDC12*, produce an hourglass-shaped collar at the bud neck between the mother and daughter cell (109) (Fig. 9). After bud emergence the "bud sensor" Elm1 and the protein kinase Hsl1 are recruited to the bud side of the septin collar and recruit the methyltransferase Hsl7 (110, 111). As a consequence, a subpopulation of Swe1 is transported from the nucleus to the neck region (112), where Swe1 is degraded. This leads to a depletion of nuclear Swe1, which promotes cycle progression because Swe1 normally inhibits Cdc28 via phosphorylation. Actin depolymerization and delayed budding activate the morphogenesis checkpoint, which stabilizes

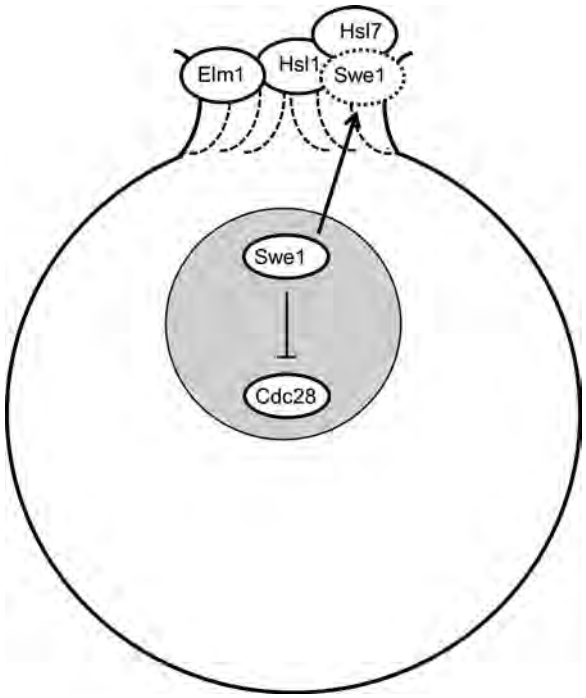


Figure 9 Summary of the morphogenesis checkpoint in *Saccharomyces cerevisiae*. In the nucleus the kinase Swe1 inhibits Cdc28 via phosphorylation. When the bud emerges, Elm1, Hsl1, and Hsl7 are recruited to the bud side of the septin collar and in turn recruit a subpopulation of Swe1 from the nucleus to the bud side of the septin collar, where it is degraded. This depletes nuclear Swe1 and allows progression into mitosis. Actin depolymerization and delayed budding activate the morphogenesis checkpoint, which stabilizes Swe1. Dotted lines represent the septin collar between mother and bud. The gray circle represents the nucleus. Swe1 with a solid border is stable and active. Swe1 with a broken line border is destabilized. The arrow shows recruitment of Swe1 from the nucleus.

Swe1 by blocking its degradation (113). Swe1 inhibits the Cdc28 CDK1 (114).

A. nidulans

Though no morphogenesis checkpoint has been identified yet in *A. nidulans*, it is possible that an analogous mechanism exists, especially in the budlike process of conidiophore formation. Conidiophore development is severely disorganized in septin deletion mutants, consistent with the possibility of a septin-dependent morphogenesis checkpoint operating at this developmental stage (115, 116).

Reboot Regulation

Classic checkpoints halt cell cycle progression when requisite events are not correctly completed to provide sufficient time for repair. But what happens when errors in cell cycle key events cannot be repaired, regardless of time? In recent work Osmani and colleagues uncovered a situation where cells appear to back up and retry cell cycle transitions multiple times, a mechanism they dubbed "reboot regulation" (117). Though reboot regulation has not yet been reported in any organism other than *A. nidulans*, it would be surprising if it was not more widespread.

S. cerevisiae

Reboot regulation has not yet been reported in *S. cerevisiae*.

A. nidulans

The eukaryotic nucleus is enclosed by a nuclear envelope (NE), which consists of an outer and an inner nuclear membrane separated by a perinuclear space (118). NPCs are large assemblies that span pores in the NE and control the nuclear transport of macromolecules across the double-membraned NE (119). *A. nidulans* undergoes a semiopen mitosis in which the NE remains largely intact, the NPCs partially disassemble, and the nucleolus is expelled from the nucleus (24, 27). After mitotic exit, functional G1 nuclei once more contain intact NPCs that gate transport and contain nucleoli.

By following NPC gating of RFP (red fluorescent protein) fused with a nuclear organization signal (NLS-RFP) and nucleolar markers, a recent study uncovered an unexpected cell cycle regulatory mechanism (117). In the absence of the inner nuclear membrane protein Src1, nuclei exit mitosis, move into G1, but then cycle back into mitosis based on NPC modifications and disassembly of nucleoli. Nuclear transport appears to be properly controlled, leading to the suggestion that there is a defect in G1 architecture. This cycle of M to G1 can repeat multiple times, indicating that the G1 nucleus is not stable. Osmani et al. coined the term "reboot regulation" to describe this cycling between M and G1 (117). They suggest that there are errors in nuclear architecture that cannot be repaired by simply allowing more time and, in these cases, the cell must dismantle and rebuild the nucleus. As the authors point out, a precedent for the cell dismantling and rebuilding incorrectly formed nuclear structures is seen in Aurora kinase action on improper spindle/kinetochore attachments. Reboot regulation promotes repetition of defective cell cycle transitions until the architectural defect is corrected (117).

CLOSING THOUGHTS

The successful cell cycle must accomplish two critical tasks: duplicating DNA and partitioning it to daughter nuclei. CDK-cyclin complexes and their regulators reliably drive the cycle forward between phases, but the timing of several critical events within the cycle are at least somewhat stochastic. The length of time it takes polymerases to synthesize new copies of DNA can be influenced by the state of DNA condensation, the supply of nucleotides, and the temperature. Similarly, kinetochore capture depends on collisions of spindle microtubules with the appropriate kinetochore region of chromosomes. It is not surprising then that surveillance (checkpoint) mechanisms have evolved to ensure that prerequisite events occur before the cycle proceeds.

We certainly have not yet found all of the surveillance mechanisms that ensure the integrity of the cell cycle. Undiscovered checkpoints seem especially likely in the case of filamentous fungi and higher organisms where nuclei must be coordinated across multiple compartments. How cell cycle signals are integrated across multiple compartments in differing developmental states is a particularly exciting question and one that studies on filamentous fungi are poised to answer.

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A Matter of Scale and Dimensions: Chromatin of Chromosome Landmarks in the Fungi

27

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CHROMATIN: AN ASSEMBLY OF DNA, PROTEINS, AND RNA

Chromosomes of fungi are linear segments of DNA, covered by a diverse assembly of RNA and proteins. They contain three landmarks required for function, namely, origins for DNA replication, centromeric DNA as attachment points for kinetochores, and telomere repeats to circumvent the end replication problem for linear DNA. Because fungi have been excellent model organisms for trail-blazing basic research since the adoption of *Neurospora crassa* as one of the workhorses for genetics in the 1940s (1), much of the foundation for general knowledge of eukaryotes was first uncovered with fungi, specifically the four species uniquely suited for genetics, biochemistry, and genomics: *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *N. crassa*. This has also been true for studies on chromatin and chromosomes.

Chromatin, the building material for chromosomes, is an assembly of DNA, proteins, and RNA, organized into nucleosome units, most of which contain octamers of canonical core histone proteins (2). The three landmarks on chromosomes mentioned above—origins of replication, telomeres, and centromeres—are characterized by different specialized chromatin modifications, for example, DNA base modifications such as cytosine or adenine methylation, and posttranslational modifications of histones; it is the set of gene silencing modifications at those chromosome landmarks that will be examined here in more detail. The occupancy of proteins binding specialized nucleosomes, and the presence of regulatory RNA species, also affects regional chromatin composition. Because this chromatin composition is generally heritable through mitosis or meiosis yet the underlying DNA base sequence is not changed, these variable chromatin compositions are considered by some as "epigenetic," acting above the DNA level to effect gene regulation, including large-scale changes in chromosome structure (2).

Since the isolation of the first histone acetyltransferase enzyme from the ciliate *Tetrahymena thermophila* (3), chromatin biology has undergone a revolution, reflecting the importance of studying transactions carried out on DNA within the normal cellular context rather than with purified DNA. In the model organisms, studies of transcriptional and posttranscriptional gene silencing, DNA methylation, cell cycle control, and the production of secondary metabolites have driven the work on chromatin structure and modification. Much of what we know about histone modification enzymes, RNA interference, and cell cycle control was first addressed in *S. cerevisiae* and *S. pombe* (4, 5). Thus, fungi have been at the cutting edge of this emerging discipline, and many recent reviews are available for exploring the details of yeast chromatin and epigenetics (4–13) or chromatin of other, mostly filamentous, fungi (14–26). One important purpose of this contribution is to suggest where additional data are necessary to better define the structure and function of chromatin and chromosomes in the fungi.

The scale of observation is important to develop a complete picture of chromatin and chromosome dynamics: are we observing a single gene, a region of coregulated genes, specific landmarks such as telomeres

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or centromeres, whole chromosomes, or the entire set of chromosomes? Scale will affect the representation of and our thinking about the segments observed, because genes or even whole chromosomes are often drawn as simple scalar plots or histograms, even though in the nucleus, chromosomes fold and interact in a nonlinear way and in three-dimensional space. High-resolution cytology (27) and sequencing-based techniques such as chromosome conformation capture (3C) followed by high-throughput sequencing (Hi-C) (28) allow unprecedented studies on fungal chromosome dynamics. Lastly, one important goal for chromatin and chromosome research in the near future will be to capture dynamic processes across time, and also at different scales, for example, milliseconds for changes in transcription upon induction by small molecules, to minutes or hours during mitosis and cytokinesis, or across a whole day when studying circadian rhythms. Under these various conditions metastable or stable rearrangements of chromatin complexes occur, some of which alter the structure of whole chromosomes in the nucleus. This is where traditional model species excel and are exceedingly useful, because of their large arsenal of methods and strains.

Of course, the kingdom Mycota is much more diverse than the current representation by a handful of models suggests. Luckily, technological advances, especially in high-throughput DNA sequencing, proteomics, and nanoscopy, make almost any organism a facile "model." Chromatin scaffold proteins, like core histones, are ancient and conserved in the fungi (29). Genes for the enzymes that modify DNA or histones are also largely conserved (14, 18, 26). One recent major discovery is the rather widespread distribution of 6-methyladenine observed in the basal lineages that is apparently absent from the Dikarya (30). Similarly, the Saccharomycetaceae can no longer methylate cytosines or adenines in DNA or methylate lysines of histone H3 at positions 9 and 27 (Fig. 1). Based on the overall phylogeny, these losses occurred more than once in different lineages, so the ancient state is predicted to be a full complement of DNA and histone modification capabilities, similar to that observed in many plants and animals. The purpose of this article is to make some of the deep knowledge garnered from a small group of organisms from restricted phylogenetic backgrounds accessible, in combination with findings from emerging model fungi, always remembering that it may be premature to derive hard and fast rules for larger groups. Ultimately, comparative biology—especially of the poorly understood chytrids and zygomycetes—will yield a much broader understanding of nature to allow us to separate the general from the specific in answering the question of how fungi assemble and maintain dynamic chromosomes.

It is far beyond the scope of this review to go into great detail on all pathways of DNA or protein modifications that can affect chromatin states. In the section on subtelomeric gene silencing we will, however, discuss two examples in more depth, namely, the histone deacetylase Sir2 and fungal homologues of the *Drosophila* histone H3K27 methyltransferase enhancer of zeste [E(z)], because they inform work in all eukaryotes.

CHROMOSOME LANDMARKS: ORIGINS, TELOMERES, AND CENTROMERES

Origins of Replication

DNA replication in eukaryotes is initiated at several loci along the chromosome, short segments called "replication origins," which are selected by binding of the origin recognition complex (ORC) (31, 32). During the late M and early G1 phase, ORC, Cdc6, and Cdt1 direct the loading of "mini-chromosome maintenance" complexes, assembling prereplication complexes that, at entry into S phase, trigger DNA unwinding and allow DNA replication. While replication origins differ in timing and frequency of activation, the maximum is only once per each nuclear cycle. While much progress has been made in a few organisms, a complete understanding of initiation efficiency and timing is still elusive, even in *S. cerevisiae*, which has been thoroughly studied (33–35). Nucleosome positioning, posttranslational histone modifications, and absence of active transcription all have some influence on the selection of ORC binding sites and replication initiation (10, 32, 36).

In some eukaryotes, including *S. pombe*, sequence requirements for replication origins are not strict, and almost any highly AT-rich sequence of ~1 kilobase (kb) is sufficient (37, 38). Specific sites are selected by interaction with the AT-hooks of the Orc4 subunit in *S. pombe* (39). This is different in *S. cerevisiae*, where specific loci function as replication origins, initially called autonomously replicating sequences (ARS), which contain consensus sequences called ARS consensus sequences that are required to allow ORC binding (40–42) (Fig. 2). Functional replication origins in *S. cerevisiae* have now been mapped by several techniques, including chromatin immunoprecipitation (ChIP) followed by genome-wide mapping via microarrays, revealing between 430 and 530 sites (33–35).

Evidence for true replication origins from most other fungi is still lacking, except for *Candida albicans* and

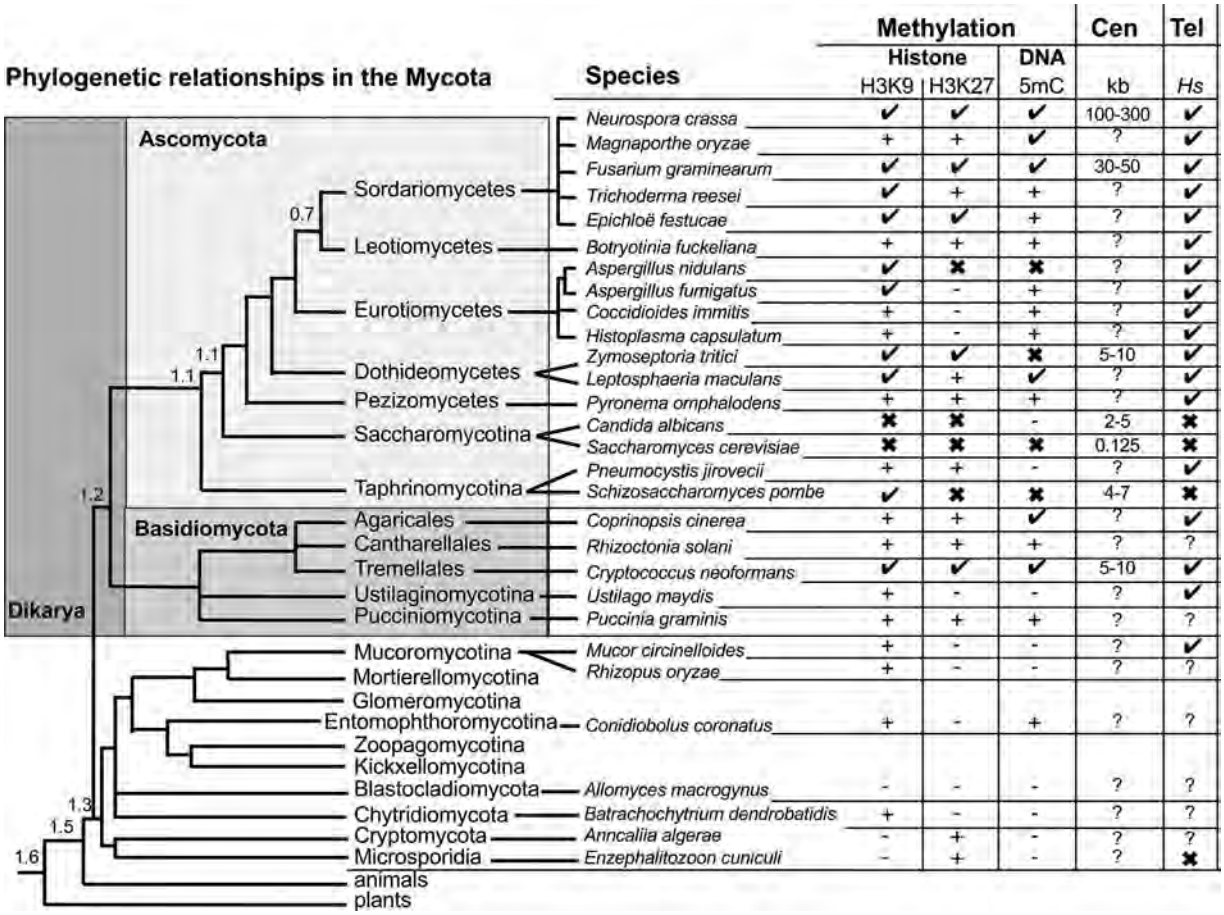


Figure 1 Presence and absence of selected histone H3 and cytosine DNA methylation marks, structure of centromeres, and sequence of telomere repeats in selected fungi. Representative fungi from various clades were selected to show the phylogenetic distribution of chromatin characteristics. Species in which the presence (check) or absence (cross) were experimentally validated are largely found within the Ascomycota, while species for which only genome sequencing-based evidence for the presence (plus) or absence (minus) of genes is available are in the Basidiomycota and the large group of early-diverging lineages. No experimental data on chromatin modifications in chytrids and microsporidia are available; some chytrids have predicted DNA methyltransferases (DNMTs) that are similar to those in animals, while some zygomycetes (e.g., *Phycomyces*) have DNMTs similar to those in ascomycetes (257). *Zymoseptoria tritici* has no cytosine methylation, but sister species have intact genes for DNMTs. No obvious DNMTs are found in the *Candida* genome, yet there have been reports on cytosine DNA methylation. *Pneumocystis*, unlike *S. pombe*, appears to have genes to carry out all modifications listed here, suggesting large diversity in the Taphrinomycotina. More recently, an entirely new class of cytosine DNA methyltransferases has been identified in fungi, demonstrating DNA methylation in species that were long thought to be devoid of methylation such as *C. neoformans* (258). The overall distribution pattern suggests that genes necessary to catalyze the two major gene silencing histone modifications, H3K9me and H3K27me, are ancient and have been lost in several branches over evolutionary time. The presence of conserved genes does not necessarily mean the presence of the expected chromatin modification. Centromeric DNA segments (Cen) are defined as regions with CENP-A or CENP-C enrichment, are highly variable in size, and even for some of the best-studied fungi such as *Aspergillus*, we still do not have experimental data. Pericentric regions, flanking the Cen regions, are larger and also of variable size. Most fungi use the mammalian and human (*Hs*) consensus telomeric repeat sequence, 5′-TTAGGG-3′, sometimes with a variable number of Gs like in *Cryptococcus*. Data on telomere repeats were compiled from the literature (54, 61, 65, 70, 72, 73, 75, 119, 124, 151, 194, 259–263).



	origins	centromere	telomere repeats
Saccharomyces cerevisiae	consensus ARS	consensus CDE	5'-$TG_{2-3}(TG)_{1-6}$-3'
Schizosaccharomyces pombe	AT-rich segments	4-7 kb core	5'-G_{0-4}GGTTACAC_{0-1}-3'
Neurospora crassa	?	100-300 kb core	5'-TTAGGG-3'
Cryptococcus neoformans	?	5-10 kb core	5'-TTAGGG-3'

Figure 2 Chromosome landmarks in four model organisms. Characteristics of DNA sequences for replication origins and centromere and telomere repeats are compared between budding yeast (*S. cerevisiae*), fission yeast (*S. pombe*), *N. crassa*, and the basidiomycete yeast *C. neoformans*. Few origins have been mapped in *C. neoformans*, so it seems premature to say whether they share specific characteristics (45). ARS, autonomously replicating sequence.

Cryptococcus. The short centromeric and pericentric regions in *C. albicans* replicate early in S phase (43) and were found to contain *bona fide* replication origins characterized by short sequence motifs (44). In *Cryptococcus deneoformans*, replication origins were mapped by enriching DNA containing replication bubbles (45). One origin was localized in the nontranscribed spacers of ribosomal DNA (rDNA) repeats. Seven additional regions on five chromosomes were identified, which—based on two-dimensional gel analyses—resemble those of *S. pombe* and humans in their relatively inefficient usage, suggesting the existence of replication zones rather than well-defined sequence elements for ORC binding (45). Note that the taxonomy and nomenclature of *Cryoptococcus* have been updated (46) and that *Cryptococcus neoformans* var. *grubii* is now referred to as *Cryptococcus neoformans*, while *Cryptococcus neoformans* var. *neoformans* is now *Cryptococcus deneoformans*.

In the other popular model species such as *N. crassa*, *A. nidulans*, and *Fusarium* species, true functional chromosomal replication origins remain to be discovered and mapped. Early attempts to find ARS in fungi outside budding and fission yeasts utilized functional approaches to isolate DNA segments—often derived from mitochondrial plasmids—to stabilize introduced DNA and to increase transformation efficiency (47–51) or used DNA segments that were preselected because of their DNA structure (52). Domestication of a transposable element, the *Aspergillus* MATE (mobile *Aspergillus* transformation enhancer), allowed the construction of a "fungal artificial chromosome" for the expression of secondary metabolite gene clusters (53), although strictly speaking, these large elements are still metastable plasmids rather than true chromosomes because no telomere sequences were captured, unlike on the *Fusarium*, *Histoplasma*, and *Cryptococcus* plasmids that have been in use but that also do not act like true artificial chromosomes (48, 54–56). ChIP-seq with a combination of ORC and mini-chromosome maintenance subunits seems to be the most obvious approach to map true chromosomal replication origins and their dynamic chromatin context, and this would close a wide knowledge gap in fungal biology.

Telomeres

Telomere repeat sequences and the shelterin complex

Telomeres were first discovered in the ciliate *T. thermophila* (57), but budding yeast soon became a facile model to uncover the structure and function of telomeres. Most organisms that have been examined use simple sequence repeats of various composition and length as a telomeric DNA sequence. In fungi, the vast majority have 5′-TTAGGG-3′ repeats, similar to the ends of mammalian chromosomes (58), but important exceptions are found within the ascomycetous yeasts in both the Saccharomycetaceae and the Taphrinomycotina (Fig. 1). One major difference between mammals and fungi is the overall length of the telomere repeat tracts, only 100 to 200 bp compared to several kilobases in most mammalian species that have been examined (59–62).

The 16 chromosomes of *S. cerevisiae* end in 75 to 300 $TG_{2-3}(TG)_{1-6}$ repeats (63), with a 12- to 15-nucleotide G-tail (64). *C. albicans* has longer, 23-nucleotide tandem repeats that are overall GC-rich and contain a *5′*-TGGTGT-*3′*; this arrangement is also found in other *Candida* species (65, 66) (Fig. 2). The canonical tandem repeat in *S. pombe* is G_{0-4} $GGTTACAC_{0-1}$ (67, 68), while the animal and human pathogens in the Taphrinomycotina, *Pneumocystis carinii* and *Pneumocystis jirovecii*, have telomere repeats composed of the more common *5′*-TTAGGG-*3′* sequence (69, 70). Telomeres from Dikarya in classes outside of the Taphrinomycotina and Saccharomycotina were found to be *5′*-TTAGGG-*3′* repeats, like in *N. crassa* (71, 72) and *A. nidulans* (73, 74); many additional species have been examined as part of genome sequencing projects (Fig. 1), though many projects did not capture any telomere repeats, e.g., in *Rhizoctonia* or *Puccinia*. There may be exceptions even in these lineages, because *Aspergillus oryzae* uses a 12-mer repeat, *5′*-TTAGGGTCAACA-*3′* (59). Direct sequence or biochemical evidence of telomere repeats is even more sparse in the early diverging lineages, e.g., among the chytrids or zygomycetes, where the standard *5′*-TTAGGG-*3′* repeat has been documented in *Mucor circinelloides* (75). Based on the genome sequences available in various databases, the microsporidia, among them *Enzephalitozoon cuniculi* and numerous *Nosema* species, appear not to have consensus telomere repeat sequences.

The telomeric DNA repeats serve as platforms for single-stranded or double-stranded DNA-binding proteins, forming protective caps or, more often, loops at chromosome ends (Fig. 3). In animals, this complex is called "shelterin" and is important for the maintenance of genome integrity, acting as a tumor suppressor involved in DNA repair (76). In fungi, analogous complexes are involved in regulated maintenance of telomere length by promoting replication at telomeres. In both *S. cerevisiae* and *S. pombe*, the telomeric repeats are free of nucleosomes and instead are organized into specialized chromatin by action of the telomere-binding protein Rap1 and two Rap-interacting proteins, Rif1 and Rif2; in *S. pombe*, the DNA-binding function of Rap1 is taken over by Taz1, and Rif2 is replaced by Rap1 (Fig. 3). As for the telomere repeat sequence, there is great diversity among telomere-binding proteins that are ill-conserved at the primary sequence level. Aside from *S. cerevisiae*, *C. albicans*, and *S. pombe*, there is still not much known about shelterin in the fungi, and mechanistic studies are sorely needed. Budding yeast and its close relatives seem to have derived protein complexes rather than representing the ancient state, whereas *S. pombe* shelterin shares functional similarities with shelterin identified in mammals (76–78). While homologues for shelterin components have been found by sequence similarity searches in many fungi (Fig. 3), it is unclear if they are also functionally conserved, illustrated by their different roles in *S. cerevisiae*, *S. pombe*, and *N. crassa* (76, 79–83).

Subtelomeric heterochromatin controls expression of disease-related genes

Segments of DNA immediately neighboring the telomeres are called "subtelomeric" and contain nucleosomes that are organized into transcriptionally silent heterochromatin in many species. Pioneering work on heterochromatin-mediated gene silencing was carried out in *S. cerevisiae* and has been reviewed recently (4, 10). Silent genes in the subtelomeric regions are intermingled with active genes; not all of the telomere-near segments are silent at all times, and silent chromatin blocks are relatively short (84). "Telomeric silencing" was first discovered by means of the *URA3* reporter gene and selection on 5-FOA medium (85). The strength of silencing is generally correlated with the length of the *5′*-TGGG-*3′* repeats, and internal telomere repeats, dispersed along chromosome arms, can aid in silencing neighboring genes in budding yeast (86). Silencing complexes restrict access to the DNA for the transcriptional machinery; i.e., switching from active to silent chromatin or *vice versa* results from a local balance between silencing and activating factors, generating so-called facultative heterochromatin.

In *S. cerevisiae*, three heterochromatic regions have been studied in great detail, namely, the silent mating type loci, subtelomeric regions, and rDNA. All three regions require the presence of the silent information regulator protein, Sir2, a conserved NAD^+-dependent histone deacetylase (4, 10, 11, 87). Sir2 is recruited to silencers or proto silencers by the action of Sir1, ORC, Rap1, and Abf1. At the silent mating type loci and subtelomeric regions, the specialized yeast Sir3 and Sir4 proteins are recruited, and spreading of this Sir complex is dependent on Sir2 deacetylase activity on H4K16 (88, 89). How exactly spreading occurs is still not resolved. The conventional idea has been that spreading of heterochromatin occurs in a linear fashion along chromosomes, though interactions *in trans* are possible, similar to some enhancer-promoter interactions. Hi-C experiments may resolve this question in the near future. Lastly, generating patches of Sir2/3/4-enriched chromatin may not be sufficient on its own to silence transcription. Several studies suggest that a

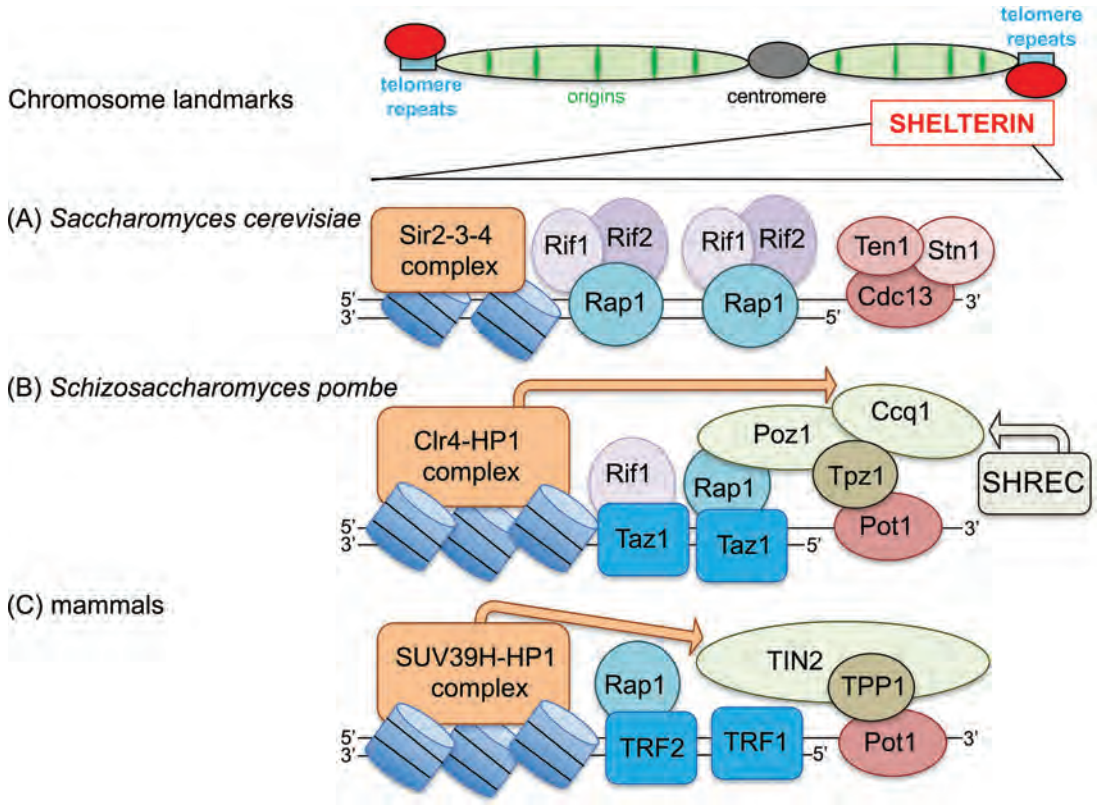


Figure 3 Telomere-repeat binding complexes homologous to mammalian shelterin. (**A**) The budding yeast *S. cerevisiae* has a CST (Cdc13, Stn1, Ten1) complex that binds to single-stranded 3′-G-rich-tail overhangs. The nucleosome-free double-stranded DNA is bound by Rap1, which in turn forms complexes with Rif1 and Rif2. Subtelomeric regions are transcriptionally silent because of hypoacetylation initiated by Sir2 and propagated by the Sir complex. (**B**) The fission yeast *S. pombe* has poorly conserved proteins serving similar functions as the CST complex, namely Pot1, Tpz1, and Ccq1. Poz1 creates a bridge to the Rap1/Taz1 complex, but Rap1 has different functions than in *S. cerevisiae*, even though there is slight sequence conservation. There is no Sir2-3-4 complex; instead, fission yeast uses H3K9me2-mediated silencing catalyzed by the Clr4 complex and recognized by HP1 (called Swi6 in *S. pombe*). (**C**) The shelterin complex first identified in mammals by purification of the first telomere-repeat factors is very similar to the *S. pombe* complex, though Ccq1 is apparently missing. In both *S. pombe* and mammals, HP1 acts on the CST complex homologues, and in *S. pombe* a histone deacetylase complex (SHREC) is involved.

"maturation" step is necessary, which may be demethylation of H3K79me3, because deletion of the sole H3K79 methyltransferase Dot1 resulted in accelerated establishment of silencing (90, 91).

In fungi closely related to *S. cerevisiae* such as *Candida glabrata*, the subtelomeric regions are enriched for genes that encode glycoprotein pathogenicity and virulence factors, similar to what has been found for parasite genomes, e.g., *Plasmodium* (92). The study of Epa adhesins, which allow binding to human cells, revealed a staggering variety in the expression of these loci near chromosome ends, and differential expression was controlled by Sir-protein-mediated transcriptional silencing (93–95). While the Sir proteins were essential, Rif1 and the Ku70/Ku80 proteins involved in nonhomologous end joining showed differential effects at only some subtelomeres (96).

During the annotation of the *C. albicans* genome, a novel family of "telomere-associated" (TLO) genes was discovered (97), which encode subunits of the Mediator coactivator complex (98–100). TLOs are involved in pathogenicity functions such as filamentous growth and biofilm formation and were considered to be adaptive by binding to specific target genes. More recent data suggest that increased protein levels of TLOs in *C. albicans* may affect regulation of virulence factors at

the transcriptional level by competition with basal transcription factors that bind to targets affecting key pathways such as filamentous growth (101). Lab evolution studies on TLO genes showed how quickly the repertoire of these genes can change; subtelomeric recombination generated diversity in copy number and sequence for TLO genes (102). Earlier work had revealed that deletion of Sir2 resulted in the rapid appearance of novel phenotypes (103), resembling (but mechanistically not related to) phenotype switching. Indeed, Sir2 is required for recombination at specific sites, called TLO recombination elements. TLO recombination elements enhance recombination, but Sir2 generally inhibits recombination, and Sir2 action can be bypassed by environmental stressors such as azole drugs and hydrogen peroxide (104).

The *S. pombe* Sir2 homologue is important for heterochromatin formation at silent mating type loci, centromeres, and subtelomeres, and it recruits Swi6 to telomeres (105). Swi6 is a homologue of the animal heterochromatin protein 1 (HP1) and binds to H3K9 that is di- or trimethylated (106). Another Sir2 homologue, Hst4, also was found to be important for subtelomeric silencing (107). In contrast to the Saccharomycetaceae, *S. pombe* uses H3K9 methylation and HP1 to induce formation of heterochromatic regions (108), and the interplay between H3K9me3 and H4K16, H3K9 and H3K14 acetylation and deacetylation has received much attention in the past decade (5). While an in-depth discussion of this topic is beyond the scope of this review, a few hallmark findings will illustrate the differences in telomeric silencing when compared to *S. cerevisiae*. Deposition of H3K9me3 and HP1 binding is dependent on the machinery that generates small interfering RNA (109), and this pathway acts at subtelomeric regions as well (110). However, Taz1 can also recruit $HP1^{Swi6}$ independently from the RNA-induced transcriptional silencing pathway (111). A Clr3 histone deacetylase complex containing the Mit1 chromatin remodeling factor ATPase, called SHREC, regulates nucleosome positioning at heterochromatic regions (112). Deletion of another telomere-associated protein, Sde2, results in decreased recruitment of SHREC and increased minichromosome formation, suggesting a role in telomere maintenance (113). SHREC directly interacts with the shelterin component Ccq1, linking Taz1, RNA-induced transcriptional silencing, and HP1-containing silencing complexes. These analyses have now come full circle, because there is accumulating evidence for the role of shelterin components Taz1 and Ccq1 in heterochromatin formation at late replicating origins that are sites for double-strand break formation during meiosis and genome organization in general (114, 115). Data obtained from high-resolution three-dimensional structured illumination fluorescence microscopy suggested that the "condensed" heterochromatin at subtelomeres in *S. pombe* is less condensed than nearby "knobs" that were eliminated by ablation of H3K36me3 (116), a histone mark correlated with gene expression and enriched in the 3′ regions of genes but actually repressing transcription from cryptic promoters and controlling transcript elongation (117, 118). Genes inserted into the condensed knobs were not silenced, whereas silent genes in the subtelomeric regions outside of the knob were less condensed, challenging our current notion of "heterochromatin" as a condensed or less accessible silent region.

In *Neurospora* there are no obvious signatures of specific gene families near the telomeres. This is different in several other filamentous fungi. Before the *Candida* virulence gene families (*epa* and *tlo*) were studied in detail, work with *Magnaporthe oryzae* had identified a family of telomere-linked RecQ-like helicases, and ectopic recombination, aided by the presence of transposons or transposon relicts, had been invoked to explain the proposed terminal truncations and reshuffling of these loci observed in this species (119, 120). In many other taxa, secondary metabolite gene clusters and genes involved in pathogenicity as "effectors," i.e., short, cysteine-rich secreted peptides or proteins, are enriched toward the telomeres, but this can encompass many hundreds of kilobases and thus is not strictly "subtelomeric silencing" (121–128).

Subtelomeric silencing has been studied in *N. crassa*, especially as it relates to H3K9me3-dependent DNA methylation and H3K27me3-mediated silencing (83, 129–137). While *S. cerevisiae* lacks H3K9, H3K27, and cytosine DNA methylation, and *S. pombe* lacks H3K27 methylation, *Neurospora* serves as an excellent model organism to decipher the roles of H3K27 and cytosine DNA methylation (23, 24), two chromatin modifications that are essential for proper development and differentiation in animals (138, 139). The *N. crassa* Sir2 homologue, NST-1, is involved in subtelomeric silencing, which was at least partially relieved when *nst-1* or three different sirtuins, *nst-2*, *nst-3*, and *nst-5*, were mutated. Even double or triple *nst* mutants, however, were not as effective in derepression of reporter genes as single deletions of HP1 or the gene for the H3K9 methyltransferase, *dim-5* (137). Because DNA methylation was only partially affected, these results suggested a DNA-methylation-independent role for H3K9 methylation in *Neurospora*. Recent studies showed that wild-type importin alpha (called DIM-3 in *N. crassa*) is

required for normal DIM-5 function (132) and for normal genome organization (131), but the mechanism for this is still unresolved.

H3K27 methylation is a hallmark of "facultative heterochromatin" in many fungi

Over the past 5 years H3K27 methylation has attracted the attention of several research groups working with *Neurospora*, *Fusarium*, *Zymoseptoria tritici*, *Epichloë festucae*, and *C. neoformans*. H3K27 methylation is catalyzed by the polycomb repressive complex 2 (PRC2), which has three core subunits: KMT6, EED, and SUZ12 (Fig. 4). The genes, E(z), Esc, and Su(z)12, respectively, were first discovered in *Drosophila* in enhancer and suppressor screens for loci affecting position effect variegation (138, 140). Specifics of the situation in *Neurospora* and *Cryptococcus* have been recently reviewed (23, 24), so the discussion here will be limited to contrasting these results with what has been found in the other three taxa.

Like the original PRC2 from *Drosophila*, NcPRC2 contains three core subunits: SET-7 (KMT6), EED, and SUZ12 (Fig. 4A); in subtelomeric regions, an additional component, NcNPF (identical to *S. cerevisiae* Msi1, *Drosophila* p55, and mammalian RbAp46/48), is required for H3K27me3 (134). The same core subunits are essential for H3K27me3 in *Fusarium graminearum*, but the Msi1 homologue, FgMSL1, is not required for H3K27me3 (L. R. Connolly and M. Freitag, unpublished results). The *Cryptococcus* $Ezh2^{EZH2}$ complex contains three conserved proteins (Ezh2, Eed, and Msl1) and two proteins unique to *Cryptococcus* (Bnd1 and Ccc1), but it lacks a recognizable SUZ12 homologue that is usually associated with E(z) (141). While there are no clear homologues of Bnd1 in ascomycetes, the best Ccc1 homologues in *Neurospora* and *Fusarium* are not involved in H3K27 methylation (130) (Connolly and Freitag, unpublished results). A partial crystal structure of the core PRC2 from *Chaetomium thermophilum*, a species of Sordariales relatively closely related to *Neurospora* and *Fusarium*, for the first time defined important functional motifs in any E(z) and EED homologue (142, 143). Allosteric interactions between KMT6 and EED had been reported previously (144), but the crystal structure and *in vitro* enzyme assays further defined interaction motifs in the H3K27me3 and H3K27 forms (143). In KMT6, 10 motifs were described (143), which are well conserved between fungal versions of the protein but are not easily discerned by sequence comparisons with proteins from animals (Fig. 4B). One clear difference in the organization of KMT6 homologues from ascomycetes is the extended sequence at the C-terminus, which is lacking from the *Cryptococcus* Ezh; the structure and function of this domain remain unknown because this segment was not included in the crystal structure. While the EED homologues all have seven WD40 motifs that form a seven-bladed β-propeller, fungal EED proteins are characterized by a long "insertion domain" (143); this is a highly variable region that is not directly involved in KMT6-EED interactions, and the function of this domain is unknown (Fig. 4C). SUZ12 homologues are overall fairly conserved when they are present in fungal genomes (Fig. 4C), though functional and structural motifs remain to be uncovered because the partial crystal structure contained only a short fragment, the VEFS motif (143).

The most obvious function of H3K27 methylation in *Neurospora* and *Cryptococcus* was proposed to be gene silencing, though loss of silencing does not result in drastic overt defects in either species (134, 141). In both species, H3K27me3 is found mostly in subtelomeric regions and a few dispersed regions on the chromosome arms, covering less than 10% of the genome (Fig. 5A). This situation is different in *F. graminearum*, where ~20% of all genes were newly turned on or upregulated when KMT6 was deleted, and where H3K27me3 covers almost a third of the whole genome (145). In *Fusarium fujikuroi*, deletion of *kmt6* is lethal, and effects of derepression of secondary metabolite gene clusters were studied by RNAi-mediated downregulation of *kmt6* (146). Again, H3K27me3 covers almost a third of the whole genome, including almost all of the smaller chromosomes (Fig. 5C), the so-called accessory chromosomes (22). In *E. festucae*, H3K9me3 and H3K27me3 in combination were reduced in secondary metabolite gene clusters that produce lolitrems and ergot alkaloids when the fungus was growing *in planta* (147). Whether the situation found in *Fusarium* and *E. festucae* is common to other pathogens needs to be resolved in the near future. There are indications that in some species such as *Leptosphaeria maculans* (148), H3K9me3 may control the activity of virulence-related genes, although the idea that long clusters of secondary metabolite genes are controlled by H3K9me3 in *Aspergillus* species may need to be readressed (149).

Studies on the interplay between H3K9 and H3K27 methylation showed that in *Neurospora*, HP1 is key for the proper distribution of H3K27me2/3 (130, 133, 150). In mutants lacking H3K9me3 or HP1, almost all H3K27me3 is mislocalized to regions previously enriched with H3K9me3, most strikingly the centromeres, and deleting components of the PRC2 at least

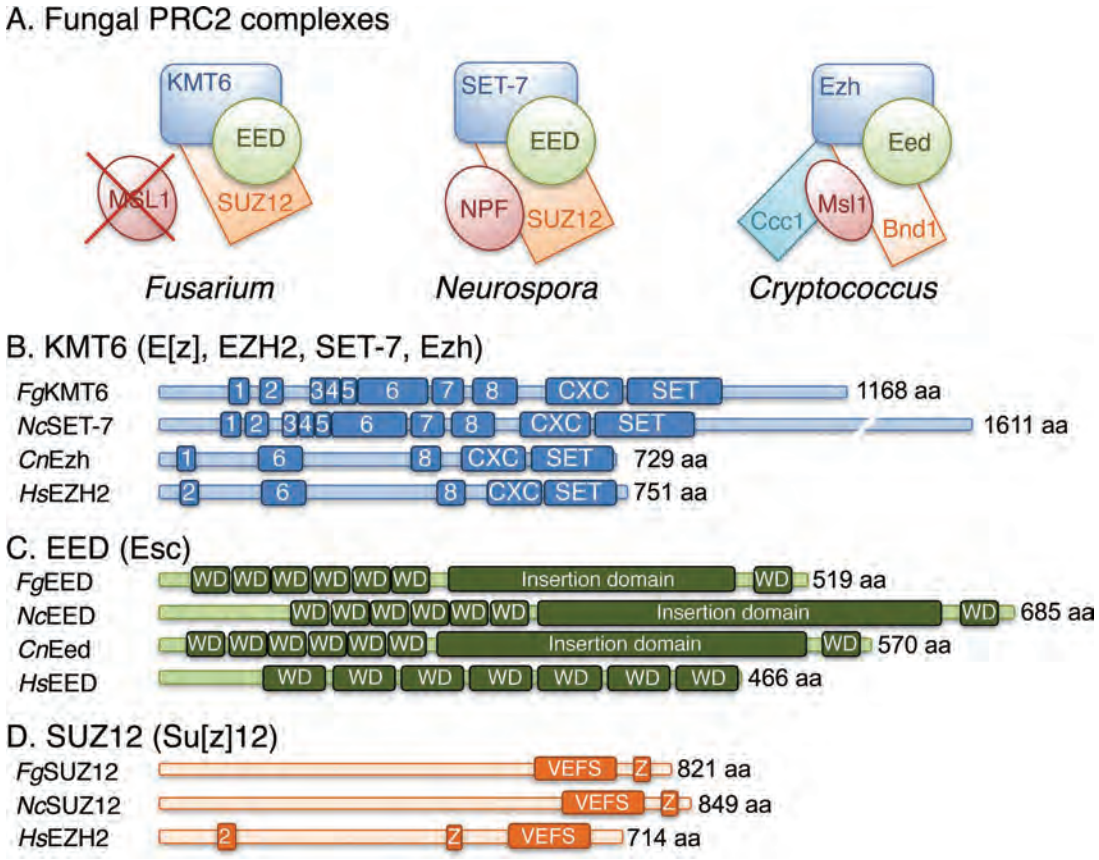


Figure 4 Polycomb repressive complex 2 (PRC2) from three fungi has different components. (**A**) Facultative heterochromatin, enriched with H3K27me2/3, is generated by PRC2 complexes. Approximate arrangement of complex subunits is based on published structures of human (264) and *Chaetomium* PRC2 (143). *Fusarium* has a core PRC2 complex that lacks a homologue of the *S. cerevisiae* Msi1 homologue (crossed out MSL1) that is found in PRC2 of *Neurospora* (NPF) and *Cryptococcus* (Msl1). While genes for KMT6, EED, and SUZ12 homologues are found in many taxa, the CnCcc1 and CnBnd1 proteins are restricted in distribution, suggesting diversification of PRC2 across the fungi. (**B**) *Chaetomium thermophilum* Ezh (*Fusarium* KMT6, *Drosophila* E(z), human EZH2, *Neurospora* SET-7, *Cryptococcus* Ezh) contains 10 structurally distinct motifs (adapted from reference 143): (i) SBD (SANT1L-binding domain), (ii) EBD (Eed-binding domain), (iii) BAM (b-addition motif), (iv) SAL (SET activation loop), (v) SRM (stimulation-responsive motif), (vi) SANT1L (SANT1-like), (vii) MCSS (motif connecting SANT1L and SANT2L), (viii) SANT2L (SANT2-like), (ix) CXC (cysteine-rich pre-SET domain), and (x) the catalytic SET domain. The SANT motifs are the least conserved surfaces in the *Chaetomium* crystal structure. (**C**) Fungal EED proteins (*Drosophila* Esc) contain WD40 (WD) domains that generate a seven-bladed propeller structure, for which the C-terminus folds back toward the N-terminus to generate propeller 1. The function of the extended C-terminal insertion domain is unknown. The accessory Msl1/NPF subunit of *Cryptococcus* and *Neurospora* is conserved in humans (RBAp46/48); all Msi1-like proteins share the WD40 propeller structure with EED. (**D**) SUZ12 [*Drosophila* Su(z)12] contains an Eed-binding domain (2), a Zn-finger region (Z), and a conserved VEFS domain that in the crystal structure is wedged between KMT6 and EED.

partially suppresses defects observed in *dim-5* mutants. In HP1 mutants, H3K9me3 and H3K27me2/3 are found in largely overlapping regions, a situation that is common in wild-type strains only in subtelomeric regions (130, 133, 150). In *Cryptococcus*, Ccc1 is involved in H3K27me3 recognition and binding, and disruption of *ccc1* results in redistribution of H3K27me3 into H3K9me2 domains, again especially the centromeric regions. If H3K9me2 deposition is abolished by deletion of the Clr4^{SUV39} homologue, H3K27me3 is

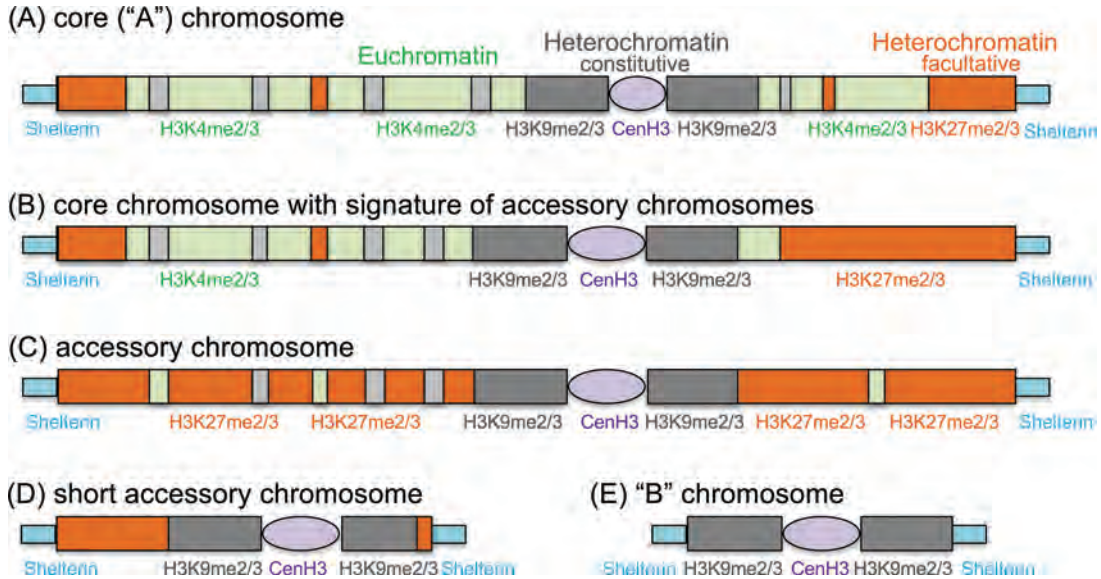


Figure 5 Histone modifications associated with transcriptionally active euchromatin and transcriptionally silent heterochromatin on four types of chromosomes found in many fungi. (**A**) Core or "A" chromosomes have a mixture of transcriptionally active euchromatin (green), constitutively silent heterochromatin (gray) that remains densely packaged even in interphase, and facultative heterochromatin (orange) that becomes transcriptionally active upon external or internal cues. Modifications on core chromosomes most often correlated with euchromatin are H3K4 di- and trimethylation (H3K4me2/3), which are usually found in sharp peaks around the nucleosome-free transcriptional start sites or in the 5′ regions of genes. In constitutive heterochromatin, which is often found in repetitive DNA sequences such as centromeric regions that also contain CenH3 nucleosomes (purple), in pericentric (dark gray) regions, or in transposable elements (light gray), H3K9 is di- or trimethylated (H3K9me2/3) and DNA is often methylated at cytosines. In facultative heterochromatin, H3K27 is di- or trimethylated (H3K27me2/3) and controls the expression of genes in a time- and space-dependent manner. Telomeric repeats (blue) have specialized chromatin structures in many fungi; some are free of nucleosomes and bound by shelterin-like complexes. In addition to the histone modifications shown here, lysines in the H3 and H4 tails of euchromatic regions are hyperacetylated (H3ac, H4ac), H3K79 and H3K36 are trimethylated (H3K79me3, H3K36me3), and H2BK120 is mono-ubiquitylated (H2BK120ub1); canonical H2A is replaced by the variant H2AZ. In heterochromatin, H3 and H4 lysines are hypoacetylated and H2AK119 is mono-ubiquitylated (H2A119ub1). (**B**) In several *Fusarium* species and in *Z. tritici*, complete chromosomes or segments of chromosome arms from accessory chromosomes that are enriched for H3K27 methylation have translocated onto core chromosomes, generating bipartite chromosomes with different histone modification environments. (**C**) Most accessory chromosomes from *Fusarium* and *Zymoseptoria* species that have been studied show almost complete coverage with H3K27me3. A very minor fraction of genes is active and enriched with H3K4me2/3, while pericentric regions and centromeric regions in *Fusarium* species are enriched with H3K9me3. In *Z. tritici*, H3K9me3 and H3K27me3 are partially overlapping in repeat-rich regions, but H3K27me3 is mostly found at silent genes. In this species no clear correlation with centromeric chromatin and any tested histone modification has been found. (**D**) The shortest accessory chromosomes have no active genes and show equal fractions of H3K27me3 and H3K9me3. (**E**) Predicted structure of true "B" chromosomes similar to those that have been found in plants and animals. These simplest chromosomes are completely gene-free and have only constitutive H3K9me3-enriched heterochromatin, centromeres, and telomeres. No such true B chromosomes have been documented in fungi.

also lost from these regions in the *ccc1* background (141). These results suggest that normal binding of the Ezh2 complex to its product, H3K27me3, via Ccc1 suppresses an inherent activity toward H3K9me2-modified centromeric chromatin regions (141). Redistribution of either H3K9me3 into H3K27me3 regions or *vice versa* has not been observed in *Fusarium* mutants (Connolly and Freitag, unpublished results). In *Z. tritici*, H3K9me3 and H3K27me3 distributions were found to overlap not only in subtelomeric regions but in most segments that are not transposons or transposon relicts (62).

In several *Neurospora*, *Fusarium*, and *Zymoseptoria* species, a correlation between subtelomeric blocks of nonsyntenic DNA and H3K27me3 has been found (62, 123, 134, 145, 146). H3K27me3 seems to act like an immune system for the fungus, perhaps silencing novel incoming DNA sequences (134), though this idea has been difficult to test, and a sequence-based "signal" for H3K27me3 targeting has yet to emerge from studies with *Neurospora* or *Fusarium*. The four chromosomes of *F. graminearum* likely resulted from chromosome fusions of the ancestral 11 or 12 chromosomes that are present in extant sister species (151). Recombination profiles (151–153) and chromatin structure analyses (145, 146) suggest that the epigenetically defined and usually silent or "cryptic" regions within chromosome arms maintain sequence diversity but also have been maintained as "subtelomeric-like regions" over millennia. What emerges from the combination of the studies discussed in the previous sections is a large variety of regulatory circuits in different taxa, suggesting that we are just beginning to uncover how and why H3K27 methylation is necessary in fungi.

Centromeres

Centromeric DNA attracts the kinetochore interaction network

Centromeres are essential chromosomal loci, dictating nucleation points for kinetochore and spindle assembly during chromosome segregation. While a handful of organisms, all related to *S. cerevisiae*, have sequence-dependent "point" centromeres that often involve as few as one or two nucleosomes, most other fungi, animals, and plants have epigenetically defined "regional" centromeres that stretch across several kilobases or even hundreds of kilobases (Fig. 1). These regional centromeres are functionally defined by the presence of a specialized histone H3 variant, CENP-A, or the presence of kinetochore complex components (154, 155). CENP-A and another essential centromere foundation protein, CENP-C, recruit the rest of the constitutive centromere-associated network, which forms the inner kinetochore, and the KNL1-MIS12-NDC80 complexes, which form the outer kinetochore and serve as the attachment point of chromatin to microtubule spindles. In combination, the constitutive centromere-associated network and KNL1-MIS12-NDC80 complexes form the kinetochore interaction network (156), where "centromere" denotes DNA and DNA-binding chromatin proteins, and "kinetochore" refers to proteins that do not directly contact DNA (157).

Compared to replication origins and telomere biology, much more is known about fungal centromeres. Among the earlier diverging ascomycetes in the Taphrinomycota, *S. pombe* is one of the best studied models. Well-defined pericentric flanking regions including the outer repeat (*otr*) and innermost repeat (*imr*) surround the central core (*cc* or *cnt*; 4 to 7 kb) (158), which contains the majority of nucleosomes with CENP-A^{Cnp1} (159). The discovery of *S. pombe* centromeres showed early on that fungi can have regional centromeres that do not depend on conserved recognition sequences for kinetochore complexes and that they can be excellent genetic models for animal centromeres (160), and by now several sister species have been studied as well (161). The poorly conserved *cc* sequences are not sufficient to allow CENP-A^{Cnp1} recruitment (162). To assemble a functional kinetochore, the *otr* and *imr* repeats or similar regions producing siRNA are required to assemble *de novo* heterochromatin marked by histone H3 lysine 9 dimethylation (H3K9me2), which is also involved in recruitment of cohesins for the binding of sister chromatids (5, 109, 163, 164). A role for centromeric repeat sequences to generate *cis*-acting short or long noncoding RNA seems to be shared by animal and plant taxa as well (165, 166).

Searches for centromere consensus sequences showed that *S. cerevisiae* has a genetically defined centromere with three conserved centromere-determining elements (CDEI, CDEII, and CDEIII) (167, 168). CDEI, an 8-bp palindromic sequence, is bound by Cbf1, and CDEIII, a conserved 26-bp motif, is bound by the CBF3 complex, interrupted by 75 to 86 bp of AT-rich CDEII sequence; Cbf1 and CBF3 are conserved only in the Saccharomycotina. This topic has recently been reviewed in great detail, and discoveries with budding yeast facilitated trail-blazing research into the requirements for kinetochore formation and "portable" centromere signals (169). Emergence of point centromeres seems to have occurred before the whole-genome duplication event that occurred in the ancestors of *Saccharomyces*, *C. glabrata*, *Naumovozyma castellii*, and *Vanderwaltozyma* (*Kluyveromyces*) *polyspora*

but after divergence of the "true" *Candida* species and *Komagataella phaffii* (*Pichia pastoris*), which have short regional centromeres (170). Thus, although sequence-specific point centromeres were discovered first, largely because of the genetic tractability of budding yeast, the combination of molecular and phylogenetic evidence suggests that point centromeres of many Saccharomycotina represent a more recently evolved state (171). One possibility is that specific sequence elements invaded chromosomes resulting in preferred binding of centromere-associated proteins and replaced ancestral regional centromeres present in the last common ancestor.

The genus *Candida* includes some of the best-studied human pathogens within the fungi, and recent work has thoroughly examined the centromeres of *C. albicans*, *Candida dubliniensis*, *Candida lusitaniae*, and *Candida tropicalis*. *C. albicans* centromeres were identified by immunoprecipitation of DNA with antibodies against CENP-A^{Cse4}, followed by cloning and sequencing (172). This direct identification of centromeric DNA showed that *C. albicans* has small (~3 to 5 kb) centromeres composed of nonrepetitive sequence elements that are not conserved, even on chromosomes of the same strain or species. Syntenic centromeric regions have different sequences in *C. dubliniensis* and *C. tropicalis* (173, 174), and centromere cores of *C. tropicalis* are flanked by inverted repeats, similar to the organization found in *S. pombe* and *K. phaffii* (173). The seven centromeres of *C. tropicalis* are more similar than those of the other species, suggesting ongoing homogenization by gene conversion. The core regions have the highest CENP-A^{Cse4} occupancy, but when (inverted) repeats are present, they are often enriched with CENP-A^{Cse4}. At the same time, *Candida* centromeres have—like *S. cerevisiae*—a single kinetochore-microtubule spindle attachment per chromosome (175, 176), thus blurring the line between point and regional centromeres.

The filamentous fungi comprise most taxa in the early diverging lineages and the Dikarya (Fig. 1). In *N. crassa*, a 16-kb region that mapped to the centromeric region of LG VII was identified in a yeast artificial chromosome library and sequenced (177, 178). The approximate sizes of all centromeres were determined after the genome had been nearly completely assembled by traditional shotgun Sanger sequencing (14, 179). Assembly of the AT-rich regions in *Neurospora* is aided by the presence of repeat-induced point mutation, a premeiotic mutator system that affects duplicated sequences as short as 150 bp (180, 181) and whose effect is heterogenization and inactivation of populations of identical retrotransposons. *Neurospora* has large (170 to 300 kb) regional centromeres, enriched with inactivated retroelements. This is similar to plants and animals, but satellite repeats are absent (182). Based on ChIP-seq with CENP-A^{CenH3}, the centromeric core regions are surrounded by short (2 to 45 kb) regions of pericentric heterochromatin. Limited examination of the *N. crassa* Mauriceville wild-type strain showed large differences in the architecture of centromeric DNA segments, initially by analysis of AT-rich segments that also had cytosine DNA methylation (183), and later by comparison of near-complete centromeric DNA sequence (184). The patterns of changes suggested that mitotic or meiotic recombination events, perhaps anchored in the near-identical repeats of nonfunctional transposons, result in large-scale insertions or deletions.

Taxa most closely related to *Neurospora* all have shorter regional centromeres, with active or incapacitated retrotransposons, similar to those found in the putative centromeric regions of *M. oryzae* (185) and *Verticillium* (127, 186). For many of these species, CENP-A^{CenH3} has not yet been mapped by ChIP-seq, though several species in the genus *Fusarium* (*F. graminearum*, *Fusarium asiaticum*, *Fusarium oxysporum*, *Fusarium solani*, *F. fujikuroi*) have been examined more closely and revealed centromeric DNA of 30 to 50 kb. All have centromeres that are enriched with retrotransposons, some of which appear to be active. Centromeres of the Eurotiomycetes, a medically and industrially important class of ascomcyetes, have still not been studied in detail, so centromeric sequences from *Penicillium*, *Aspergillus*, *Histoplasma*, and *Coccidioides* remain unknown or unassembled. In *A. nidulans*, repeat elements, e.g., the *Dane1* and *Dane2* long terminal repeat elements, have been found (187), and based on comparative genomics, centromeric regions are thought to be between 8 and 80 kb long (188). Like in *Candida*, centromeres of the sister species are embedded in regions with high synteny, even though centromeric sequence has diverged. In the Dothideomycetes, the location of centromeres had been predicted based on the longest AT-rich regions on each chromosome. Surprisingly, such predictions turned out to be wrong for the genus *Zymoseptoria*, in which most centromeres are not associated with AT-rich DNA (62). Instead, ChIP-seq with *Z. tritici* CENP-A^{CenH3} revealed short (5 to 10 kb) CENP-A-enriched regions without distinct sequence patterns; some centromeres contain expressed genes, while others harbor active or silent retroelements.

Among the Basidiomycota, only *C. neoformans* has been examined in any detail for centromere sequences,

both by sequence comparisons and ChIP-seq with CENP-C (45, 189). Together, the pericentric and centromeric regions are between 20 and 65 kb long and enriched for active or disabled Tcn1-Tcn6 retrotransposons. Mapping of CENP-C showed that this centromere foundation protein binds to a core region within the centromeric and pericentric region, covering ~5 kb on *CEN14*. The flanking regions found in *C. neoformans* and *C. deneoformans* are mostly syntenic, but the chromosome numbering is different for the two subspecies; a similar arrangement was observed with the more distantly related *C. gattii* (45). The well-studied *Ustilago maydis* seems to have short centromeres that are associated with ARS (190, 191), but again, no detailed studies have been undertaken.

The last common ancestor of fungi split from the animal lineage ~1.5 billion years ago, and the most basal fungal lineages, the Microsporidia and Cryptomycota, separated from the precursors of the zygomycetes, Ascomycota and Basidiomycota, ~1.3 billion years ago (Fig. 1) (192, 193). No molecular studies have investigated the position of centromeres in the basal taxa, and standard genome sequencing alone will likely prove insufficient because it is unclear that all centromeres are in long AT-rich regions. The genome of the industrially important *Trichoderma reesei* has been assembled by 3C followed by Hi-C (194). This method makes no *a priori* assumptions about centromere clustering (131), so application of Hi-C-aided assembly allows the precise mapping and assembly of centromeres of many fungi. While this method will provide important information, the extent of centromeres should always be confirmed by localization of the defining epigenetic marker, CENP-A.

What emerges from investigations on short point or extended regional centromeres in the fungi is the realization that genes surrounding the centromeric core or repeat regions of related species are syntenic, while the core regions are highly divergent. This suggests that centromeric sequences undergo mutation without repair at a higher frequency than surrounding sequences. It remains to be determined whether this implies positive selection toward highly adapted sequence signals aiding deposition of CENP-A (195, 196) or, rather, genetic drift because CENP-A deposition is determined entirely epigenetically by pre-existing CENP-A nucleosomes, thus making DNA sequences immaterial.

Centrochromatin

That centromeric chromatin is different from bulk chromatin became clear when CENP-A was identified as a histone H3 variant (197–199). The sparsity of genes, enrichment with repeat elements and active or disabled retrotransposons, transcriptional repression, and compaction during interphase pegged centromeric chromatin as constitutive heterochromatin, but this idea was challenged by results that showed the presence of histone modifications commonly associated with active transcription in fission yeast, *Drosophila*, and human cells (110, 200, 201). Thus, along with transcriptionally active euchromatin and silent heterochromatin, "centrochromatin" may constitute a distinct, third form of chromatin.

CENP-A is rapidly evolving, especially the N-terminal tail, loop 1 of the histone fold domain, and the CENP-A targeting domain, which are all involved in CENP-A localization and kinetochore interactions. In fungi, this has been studied in the Taphrinomycotina (202), Saccharomycetaceae (203), and Sordariomycetes (P. Phatale, S. Friedman, and M. Freitag, unpublished results). The C-terminal tail is important for recruitment of two essential constitutive centromere-associated network components: CENP-C and CENP-N (204–207). The controversy about the shape and size of centromeric nucleosomes across the cell cycle has been reviewed (169), and the balance of CENP-A nucleosomes and posttranslationally modified H3 nucleosomes is a topic of ongoing investigations; earlier studies uncovered roles for histone modifications in *de novo* establishment of stable fission yeast centromeres (162). How histone modifications may aid in centromere maintenance, however, remains to be uncovered in most organisms. Because the complement of histone genes is very simple in fungi—there are single genes for H2A, H2B, and H3 and only two genes encoding identical H4 proteins (208)—and because most fungi are genetically and molecularly tractable organisms, this may be the area where they can contribute the most to advances on centrochromatin in the near future.

Centromere inactivation or deletion experiments in *C. albicans* demonstrated the utility of this system for the study of "neocentromere" formation and inheritance (209). Neocentromeres are previously naive chromatin regions that give rise to functional kinetochores competent for chromosome segregation. Upon inactivation or deletion of the original centromeres, *Candida* neocentromeres form almost anywhere on the chromosome, though there is some preference for transcriptionally silent pericentric and subtelomeric regions (43, 210–212). Like in *S. cerevisiae*, centromeres and neocentromeres in *Candida* interfere with transcription of nearby genes (210), also suggesting heterochromatin characteristics. The role of histone modifications for centrochromatin in *Candida* is unresolved;

like *S. cerevisiae*, *Candida* lacks the conserved heterochromatin pathways that rely on methylation of H3K9 and H3K27.

Constitutive heterochromatin, defined in many eukaryotes by the presence of H3K9 di- or trimethylation (H3K9me2/3) and cytosine DNA methylation, is also a characteristic of most regional pericentric or centromeric regions. In *S. pombe*, the RNAi machinery is essential to recruit H3K9me2 to the pericentric repeat and help to incorporate CENP-A^{Cnp1} on naive plasmid sequences (162). Once assembled, however, the heterochromatin machinery, including the histone methyltransferase Clr4^{SUV39} and the H3K9me2 adapter protein Swi6^{HP1}, is no longer required for CENP-A^{Cnp1} inheritance. Nevertheless, heterochromatin is essential for proper chromosome segregation and chromosome structure, likely by the recruitment of cohesins (163, 213–215). Early genome-wide ChIP studies showed that the *imr* and *cc* (*cnt*) regions were enriched for a euchromatic mark, H3K4me2 (110), but recent experiments suggest that H3 nucleosomes are depleted from the centromeric core and that CENP-A^{Cnp1} nucleosomes and the CENP-T complex dominate (159).

There have been far fewer studies on centrochromatin of other clades in the fungi. Centromeric regions of *N. crassa* contain blocks of canonical nucleosomes that are enriched with H3K9me3 interspersed with blocks of CENP-A^{CenH3} nucleosomes (182, 216). In mutants lacking the H3K9me3 methyltransferase DIM-5^{SUV39} and the H3K9me3-binding protein HP1, the regions enriched for CENP-A^{CenH3} were smaller. H3K4me2/3 did not associate with the formerly H3K9me3-enriched nucleosomes, though overall nucleosome occupancy seemed unaltered (182). Further studies revealed relocalization of H3K27me2/3 to regions usually occupied by H3K9me3 in DIM-5^{SUV39} and HP1 mutants, suggesting that HP1 prohibits H3K27 di- and trimethylation in H3K9me3 regions (130, 133). Single mutants lacking DIM-5^{SUV39} and HP1 show growth and chromosome segregation phenotypes and are homozygously sterile or result in aberrant progeny (217, 218), while SET-7^{EZH2} mutants show no overt defects (134). In DIM-5^{SUV39} SET-7^{EZH2} or HP1 SET-7^{EZH2} double mutants, these phenotypes are largely suppressed (130, 133), suggesting that it is the presence of H3K27me2/3 at centromeres or its absence from normal facultative heterochromatin that results in the chromosome segregation defects.

A link between the RNAi and meiotic silencing pathways and heterochromatin establishment in *N. crassa* has not been found (219), unlike in fission yeast. In *F. graminearum*, centrochromatin is similar to *Neurospora*, although the relocalization of H3K27me2/3, which has been observed in *Neurospora* DIM-5^{SUV39} and HP1 mutants (130, 133) as well as *C. neoformans ccc1* mutants (141), does not occur in *F. graminearum* (Connolly and Freitag, unpublished results). Overt phenotypes are also drastically different from those observed in *Neurospora* (134), because H3K9me3-defective strains have no discernible phenotypes under standard growth conditions, while single mutants lacking H3K27me3 or double mutants lacking H3K9me3 and H3K27me3 show numerous developmental and other defects (145). In *Z. tritici*, centrochromatin cannot easily be defined because CENP-A-enriched regions do not show any obvious DNA sequence or chromatin pattern (62). So far, only H3K4, H3K9, and H3K27 methylation have been tested, but none of these marks overlap reliably with CENP-A localization. The only basidiomycete whose centrochromatin has been studied is *C. neoformans* (45, 189). H3K9me2 is found almost exclusively in the centromeric, pericentric, and subtelomeric regions. Overall, fungi represent diverse opportunities to test centrochromatin plasticity that is still difficult to carry out in many other organisms. All known variants of centrochromatin in fungi are more similar to heterochromatin than euchromatin, whether due to the presence of silencing marks (e.g., H3K9me2/3) or the involvement of the RNAi machinery in *S. pombe*.

NOT ALL CHROMOSOMES ARE EQUAL

How To Tell Core from Accessory Chromosomes

Many eukaryotes carry extra "B" chromosomes in addition to the set of core ("A") chromosomes (220). By definition, B chromosomes are not essential; most are harmful to the host, and at best they are neutral elements. In fungi, however, an overwhelming majority of B chromosomes seems to confer benefits by carrying genes involved in plant or animal colonization or to be involved in detoxifying host defense compounds, explaining the term "pathogenicity" chromosomes (221, 222). Selective advantages through pathogenicity determinants do not hold for all of these extra chromosomes, however (122, 223). In some fungi, B chromosomes are found only in specific accessions of one species, hence the name "lineage-specific" chromosomes (221); other monikers are "conditionally dispensable," "supernumerary," or "accessory" chromosomes (224–226).

Animal and plant B chromosomes use various forms of meiotic drive and self-accumulation mechanisms to

propagate, and many of the inheritance patterns are non-Mendelian. While there is much variation, some rules seem to apply (220, 227, 228). The more B chromosomes there are in a single nucleus, and the harsher the environment, the more pronounced are the negative effects of B chromosomes. B chromosomes can affect recombination frequency on A chromosomes, and in plants, inbreeding promotes the accumulation and spread of rare beneficial B chromosomes. Overall, the presence of B chromosomes is positively correlated with low ploidy, low chromosome numbers, and large genomes (and therefore much larger domains of repetitive DNA). B chromosomes are also more prevalent in genomes with acrocentric chromosomes, and smaller B chromosomes are mitotically less stable than large ones (220, 227, 228). In fungi, the rule seems to be that accessory chromosomes are rarely larger than the smallest A chromosome, and this is true in most eukaryotes. B chromosomes are small, containing sometimes only the elements required for propagation, i.e., telomeres, centromeric DNA for kinetochore and spindle attachment, and origins of replication, but there is a large diversity in B chromosomes in plants and animals.

So what separates accessory and B chromosomes? The chromosome structure of *Fusarium* and *Zymoseptoria* species has been studied extensively, because they have a large number of accessory chromosomes (62, 224–226). Overall, accessory chromosomes are depleted for genes and enriched for repeated elements, mostly active and mutated transposons that are shared or rarely of different types than on the core chromosomes (221, 225, 229). Very few—if any—genes have been found on B chromosomes in plants or animals; it appears that an increase of gene density on accessory chromosomes is thus positively correlated with benefit to the host organism. Matching the finding of few active genes, cytological data from plants and animals suggest that B chromosomes are heterochromatic, and most studies suggest that they are enriched for H3K9me2/3 (230, 231). In fungi, the hallmark chromatin feature of accessory chromosomes is H3K27me3 (22, 62, 145, 146), though transposable elements are still covered by H3K9me3 in *Fusarium* and *Z. tritici* (Fig. 5). Thus, the separating characteristic—at least for now—seems to be the presence of H3K27me3 and facultative heterochromatin on accessory chromosomes.

How Do B Chromosomes or Accessory Chromosomes Arise?

Plant and animal B chromosomes are likely predominantly generated within the host by aberrant chromosome segregation during division; "horizontal chromosome transfer" (HCT), similar to horizontal gene transfer in bacteria but involving entire chromosomes, is considered a less likely pathway (220). The finding that species with acrocentric chromosomes are more likely to generate B chromosomes supports this idea, providing a mechanism that suggests breakage within centromeric DNA regions and addition of telomere repeats to generate a minichromosome that can be segregated in mitosis. This may be inefficient, however, especially when centromeric regions are insufficient for optimal kinetochore and spindle attachment, resulting in stochastic inheritance of B chromosomes, which may result in extinction at accelerated rates (220).

For fungi, HCT has been long considered as one model for the acquisition of accessory chromosomes, but HCT may mean different things to scientists from various disciplines. Most authors have made no distinction about species boundaries when discussing HCT (221, 222, 232–235), using "horizontal" or "lateral" transfer merely to describe a process distinct from the "vertical" transfer through the germline. Others may argue that transfer of new chromosomal material has to occur between different species before it is truly horizontal. This very specific condition is only rarely fulfilled; even horizontal gene transfer between species is difficult to detect, although there are some cases in which genes encoding host-specific toxins have been transferred (236). The hurdles for HCT even within species are high; no satisfying mechanistic explanation for how this may occur in *Fusarium* or *Alternaria* has been conclusively validated.

Chromosomes with drastically altered gene sequences, transposon or repeat content, and codon bias that may no longer resemble those commonly found in the same species have been observed in some species (221, 225, 237). Interspecific hybridization may occur between fungal genera, yet endogenous sources for the generation of accessory chromosomes seem more likely, especially in light of recent results obtained with *Fusarium* and *Zymoseptoria*. While HCT has been shown to occur within the "species" *F. oxysporum* between different *formae specialis* under strong selection (221, 238, 239), this is more difficult to show for different species or across different genera. The emerging consensus is that accessory chromosomes in *F. oxysporum* are not only "lineage-specific" but appear to be *bona fide* pathogenicity factors because of the reliable conversion of nonpathogenic strains into pathogenic strains (240–244). How many traits or specificity factors can be assembled into one such chromosome?

One wonders whether one pathogenicity gene or set of pathogenicity genes on each accessory chromosome is specific to only one host. In other words, are pathogenicity chromosomes inherited as a single trait?

Studies with *Fusarium* and *Zymoseptoria* also showed that core chromosomes with large segments of facultative chromatin typical of accessory chromosomes exist (62, 221). These cases suggest that either subtelomeric H3K27me3-enriched regions increase and spread to encompass longer segments, or—perhaps the more likely scenario—translocations between accessory and core chromosomes occur on a regular basis (Fig. 5B). Some evidence for the second idea is provided by mapping of *Z. tritici* chromosome 7, where the boundary between core and accessory chromosome characteristics falls almost precisely in the regions where the rDNA cluster was mapped (62, 245). This suggests that there may be preferred regions for reciprocal or nonreciprocal translocations, which predominantly seem to be linked to H3K9me3-enriched transposable elements (62, 126).

While intraspecies HCT and translocations from preexisting accessory chromosomes can explain some of the variation within a species, these mechanisms still do not explain how relatively gene-rich fungal accessory chromosomes arise in the first place. Endogenous generation of a novel chromosome from two existing accessory chromosomes by chromosome fusion followed by degenerative breakage, as proposed by the "breakage-fusion-bridge" model (246, 247), may serve as a general explanation for how accessory chromosomes in *Z. tritici* arise (226). Repetitive DNA may result in nonallelic homologous recombination between repeats to generate a dicentric and an acentric chromosome. The dicentric chromosome (in the case of *Z. tritici* chromosome 17) may have undergone breakage-fusion-bridge cycles (226), while the acentric chromosome was simply lost in subsequent divisions. There are indications that chromosome loss in this species is quite common (223), especially in meiosis where short chromosomes may not support the necessary cross-over events for successful segregation (248).

Successive cycles of translocations or breakage-fusion-bridge may result in chromosomes that are successively more enriched in H3K27me3 facultative heterochromatin and depleted in H3K4me2/3 euchromatin (switch from panel B to C in Fig. 5), and further degeneration of chromosomes may result in extremely short accessory chromosomes that have a mixture of H3K27me3 and H3K9me2/3 marks but no longer carry any essential genes that would be enriched with H3K4me2/3. If driven to the extreme, this may result in true B chromosomes that carry only H3K9me2/3 (Fig. 5E), like in some plant and animal species, though this type of chromosome has not yet been observed in fungi.

CHROMATIN INTERACTIONS WITHIN THE WHOLE NUCLEUS

On the previous pages we examined landmark regions required for chromosome function in a reductionist fashion, but the pieces must work together. There are various ways to examine chromosome dynamics, one being cytology (4, 12), which has now entered into the age of high-resolution "nanoscopy" (249). Another way to resolve contacts between chromosomal regions at high resolution is 3C followed by Hi-C (28, 250) and its many variants (251). Several studies of budding and fission yeast, as well as *N. crassa*, resolved the importance of chromosome landmarks for normal growth in fungi. Hi-C and cytological studies with wild types and mutants defective in constitutive (H3K9me2/3-enriched) or facultative (H3K27me2/3-enriched) heterochromatin showed that, like in budding (252) and fission yeast (214, 253), all centromeres of *N. crassa* are colocalized within the nucleus, revealing Rabl orientation (129, 131). Surprisingly, all mutants that disturb features of transcriptional silencing, or "heterochromatin," namely those that are lacking HP1, the H3K9 methyltransferase DIM-5 (i.e., KMT1, SUVAR39), or the H3K27 methyltransferase SET-7 [i.e., KMT6, E(z)] revealed only minor changes in overall chromosome organization (Fig. 6). Cytology showed altered position and increased numbers of centromere foci in the SET-7 but not the DIM-5 and HP1 single or *set-7*, *dim-5* double mutants (129). These data suggest a role for H3K27me2/3 in the control of centromere maintenance. In fission yeast, cohesins and condensins seem to play a major role in maintaining overall chromosome structure (13, 214, 254). Recent studies revealed surprising roles of condensins in organizing centromeric regions in fission yeast (255, 256). Thus, Hi-C studies suggest that there is higher-order organization to chromatin, though the precise role of heterochromatin, cohesins, condensins, or other scaffolding proteins is still uncertain.

In summary, while it is clear that chromatin modification enzymes are important for the establishment of large chromatin segments, more work must be done to determine what, if any, negative effects on chromosome segregation occur in a large number of chromatin mutants. Cytology has been carried out for a long time, but 3C-based studies carried out across the cell cycle in synchronized cells will be necessary to provide a much

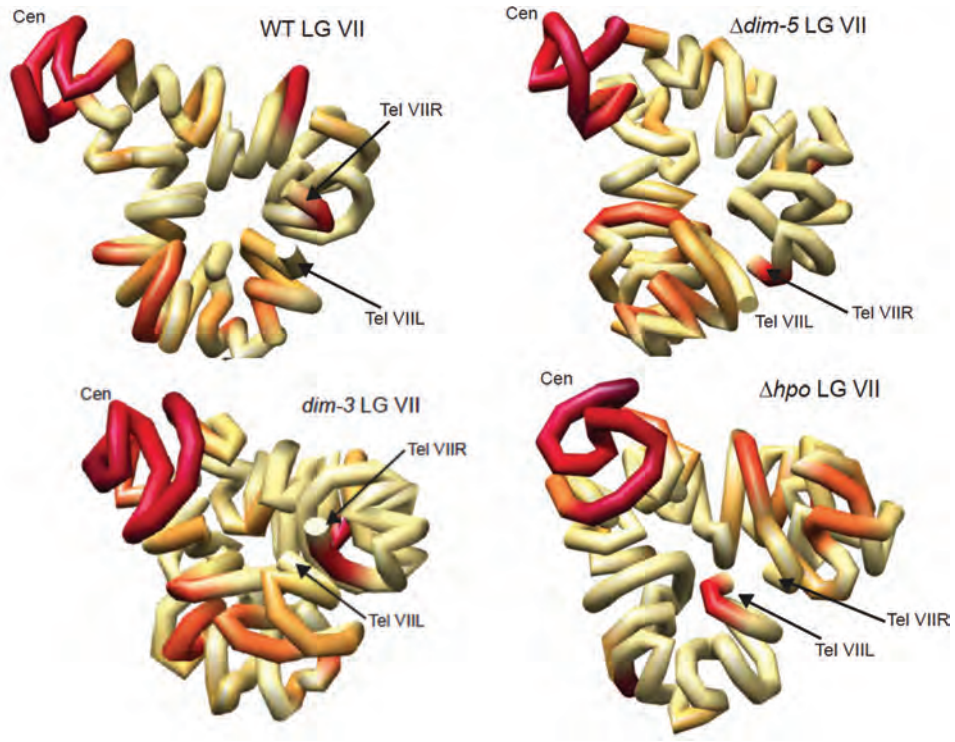


Figure 6 Three-dimensional models of *N. crassa* linkage group (LG) VII based on Hi-C data. Chromosomes are represented as wire diagrams, where the wire path runs through the center of a series of 50-kb "spheres" determined by the contact frequencies calculated from Hi-C datasets for the wild type and three chromatin mutants (*dim-5*, *hpo*, *dim-3*). The chromosome path is calculated by attractive or repulsive forces between each sphere so that the system relaxes to a low energy state. Regions that are enriched with one heterochromatic mark, H3K9me3, in the wild type are shaded in red. Centromeres and subtelomeres are separated, but telomeres are closer to each other than to the centromere (adapted from reference 131).

deeper understanding of the relationships between chromatin state and chromosome landmark functions.

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Ploidy Variation in Fungi: Polyploidy, Aneuploidy, and Genome Evolution

28

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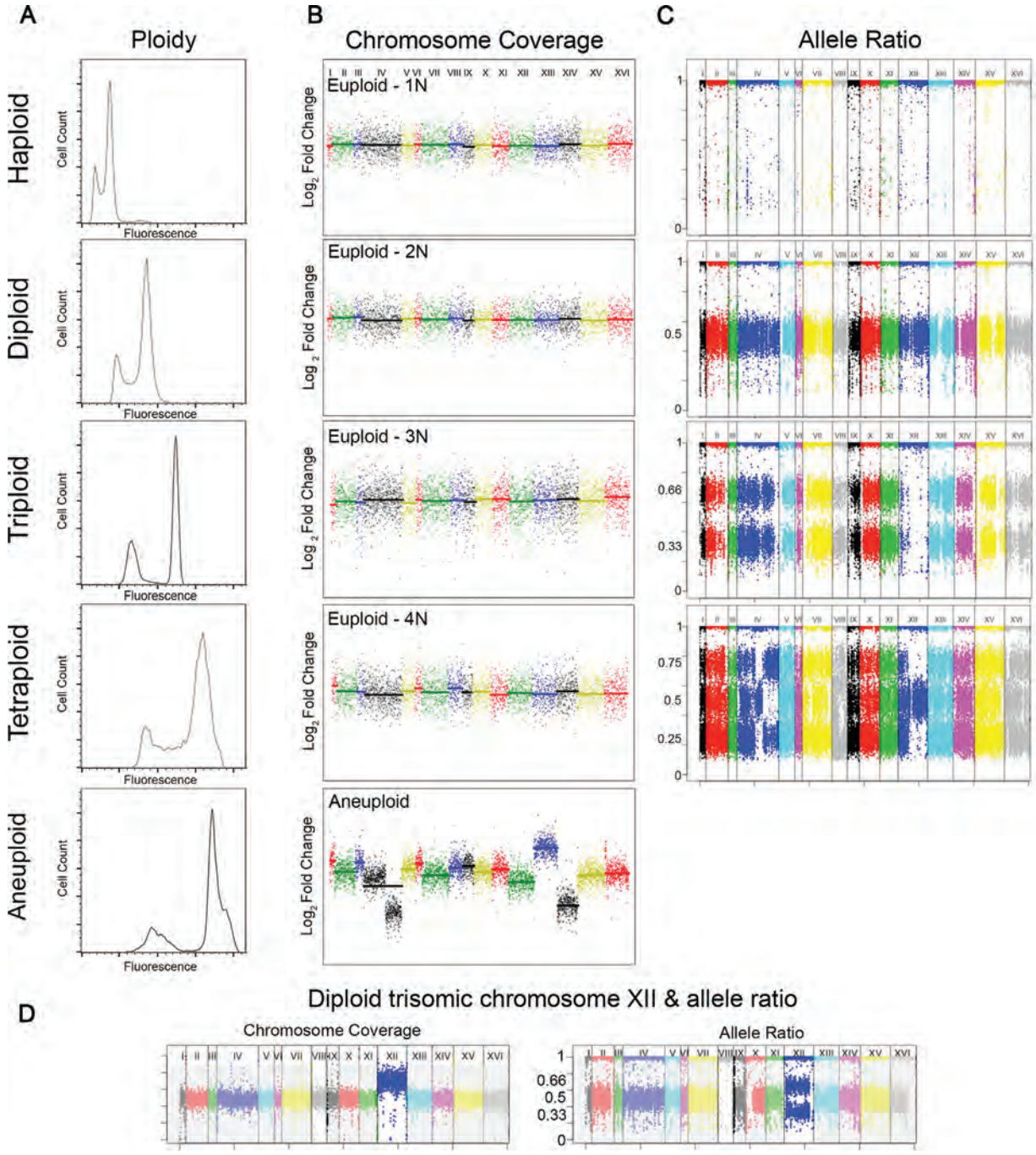
INTRODUCTION

Cellular ploidy is the number of complete sets of chromosomes in a cell. Many eukaryotic species have two (diploid) or more than two (polyploid) sets of chromosomes (1). These diploid and polyploid states are often the result of ancient whole-genome duplication (WGD) or hybridization events that occurred throughout the evolution of plants, animals, and fungi (2–4). Ploidy changes also occur during the development of many organisms and can vary within different tissues of the same organism and between individuals of the same species. For example, ploidy changes occur during the sexual cycle of eukaryotes, from haploid gametes to diploid somatic cells. Additionally, some cells continue to increase in ploidy during development, resulting in somatic tissues that have a mixture of diploid and polyploid cells, including human hepatocytes and megakaryocytes (5–7). These ongoing, developmentally programmed changes in ploidy are important for viability and are beneficial to many organisms (8), but the mechanisms controlling ploidy and the physiological significance of each ploidy level are not well characterized.

Many clinically relevant fungi undergo ploidy changes during adaptation, especially to adverse or novel environments. Some fungi exist as stable haploid, diploid, or polyploid (e.g., triploid, tetraploid) cells, while others change ploidy under certain conditions and revert back to the original ploidy level in other conditions. Aneuploidy, an abnormal chromosome number, is observed in novel environments, during periods of cellular stress, and during ploidy level changes. An increase in ploidy can occur through mating, endoreduplication, or failure to undergo cytokinesis after replication (described in detail below). Here we refer to events that increase cellular ploidy as WGD, diploidization (e.g., haploid to diploid), or polyploidization (e.g., diploid to tetraploid). A decrease in fungal ploidy can occur through meiosis, reductional division, and random chromosome loss events (9). We will discuss only nonmeiotic ploidy decreases and refer to these events as whole-genome reduction events or haploidization events (e.g., diploid to haploid). The examples we provide here challenge the textbook definition of somatic ploidy as a consistent or defining trait of a fungal species. Instead, fungal ploidy is often context-dependent and can rapidly change from one environment to the next.

Ploidy values currently assigned to fungal species may be influenced by laboratory growth conditions and selection for traits that prove to be beneficial for conducting genetic manipulations. However, as whole-genome sequencing (WGS) and molecular assays for ploidy detection become standard in fungal research labs, the identification of ploidy variants is increasing. For example, polyploid strains of the typically haploid and/or diploid species *Saccharomyces cerevisiae*, *Candida albicans*, and *Cryptococcus neoformans* exist in diverse environments including desert canyons, fermentation and industrial cultures, and from human patients before and after therapeutic treatment with antifungal drugs (10–16). This suggests that many different ploidy-environment interactions may select cells with increased or decreased ploidy that provide these cells with an adaptive advantage (1, 17, 18). A mechanistic understanding of what drives environment-dependent ploidy changes remains to be discovered. Genome sequencing and phenotypic characterization of mutations in these comparatively small eukaryotic genomes (e.g., compared to human genomes) will likely place fungi at the forefront of ploidy research (19–21).

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A
Ploidy
Haploid
Diploid
Triploid
Tetraploid
Aneuploid
Cell Count
Fluorescence
B
Chromosome Coverage
Euploid - 1N
Euploid - 2N
Euploid - 3N
Euploid - 4N
Aneuploid
Log2 Fold Change
C
Allele Ratio
D
Diploid trisomic chromosome XII & allele ratio
Chromosome Coverage
Allele Ratio

Molecular Detection of Ploidy and Aneuploidy

Ploidy is most commonly measured by flow cytometry of fluorescently labeled cells (e.g., propidium iodide), where the relative fluorescence of an unknown isolate is compared to strains of known ploidy (22). More than 30,000 single cells can be analyzed within seconds, and linear increases in ploidy are detected with great accuracy (Fig. 1A). Aneuploid isolates are detectable by flow cytometry with the caveat that isolates with a single aneuploid chromosome may not be significantly different in the fluorescent signal compared to the known ploidy control. In addition, the fluorescent signal of isolates with multiple aneuploidies (e.g., gain of one and loss of another chromosome) may not show different DNA fluorescence by flow cytometry because these specific aneuploidies cancel each other out. Instead, more quantitative methods must be used to identify the specific aneuploid chromosomes. Flow cytometry coupled with additional molecular methods such as comparative genome hybridization (aCGH), quantitative PCR, and double-digest restriction site-associated DNA sequencing (ddRADSeq) is the most comprehensive approach to detect ploidy changes and identify specific aneuploidies (23–25).

The ploidy level and chromosome copy number of isolates can also be determined using WGS. The copy number of each chromosome is determined relative to the entire genome based on the number (depth) of aligned sequence reads. Aneuploidy is detected as an increase or decrease in read depth relative to the entire genome (Fig. 1B). Segmental chromosome aneuploidies and small gene amplifications/deletions are also detected. Additionally, WGS bioinformatics tools use allele frequencies to determine the baseline ploidy of the sequenced genome (16, 26). For example, a haploid (1N) genome has allele frequencies at 1, a diploid (2N) genome has allele frequencies at 0.5 and 1, a triploid (3N) genome has allele frequencies at 0.33, 0.67, and 1, and a tetraploid (4N) genome has allele frequencies at 0.25, 0.5, 0.75, and 1 (Fig. 1C and D). While limited to strains with significant heterozygosity, WGS simultaneously detects cellular ploidy level, chromosome copy number, and sequence polymorphisms. Further studies are needed to truly understand the extent of ploidy variation in natural populations within and between species.

Ploidy Variation in Natural Isolates

We first highlight three examples of ploidy variation found in isolates of *S. cerevisiae*, *C. neoformans*, and *C. albicans*. These three species have different genome sizes, haploid chromosome numbers, sexual cycles, and preferred base ploidy levels. Despite these differences,

Figure 1 Methods for detection of ploidy and aneuploidy. (**A**) Ploidy is determined with flow cytometry. Total genome fluorescence, measured using a fluorescent nucleotide label (e.g., propidium iodide or Sytox Green). Cells are first fixed (in ethanol) and RNA is removed with RNase, then genomic DNA is fluorescently labeled and analyzed on a flow cytometer. Cells are passed through a laser, and the number of cells are plotted as a function of fluorescence intensity. Cells in a population typically have two fluorescent peaks, representing cells in either G1 or G2 phases of the cell cycle. Flow cytometry plots for yeast with the following ploidy levels are shown: haploid (1N), diploid (2N), triploid (3N), tetraploid (4N), and a near-tetraploid aneuploid. (**B**) Chromosome copy number is determined with WGS and microarray aCGH. The *y* axis represents a $\log_2$ fold change of sequence reads relative to the reference sequence and chromosome number increases from left to right starting with chromosome I and ending with chromosome XVI (*x* axis). Chromosome copy number plots for *S. cerevisiae* with the following ploidy levels indicate euploid genome for haploid (1N), diploid (2N), triploid (3N), and tetraploid (4N). However, the near-tetraploid isolate (bottom panel) is aneuploid for ChrXII (pentasomic) and ChrXIV (trisomic) and contains a segmental aneuploidy of ChrIV. Figures generated from data obtained in reference 20. (**C**) Allele frequencies obtained from WGS data also can be used to determine the ploidy of a strain. The *y* axis shows the heterozygous allele frequencies ranging from zero to one, plotted as a function of chromosome number starting with chromosome I and ending with chromosome XVI (*x* axis). Allele frequency plot of the example haploid strain with single nucleotide polymorphisms (SNPs) at allele frequencies at 1.0; a diploid strain with SNPs at allele frequencies of 0.5 and 1.0; a triploid strain with SNPs at allele frequencies of 0.33 and 0.66; and a tetraploid strain with SNPs at allele frequencies at 0.25, 0.5, 0.75, and 1.0. Images obtained from reference 16. (**D**) A diploid strain that is trisomic (three copies of a chromosome) for chromosome XII (left panel). Interestingly, the allele frequency plot has SNPs at allele frequencies of 0.5 and 1.0 for all chromosomes except ChrXII, which is at allele frequencies of 0.33 and 0.66, supporting that this chromosome is aneuploid (right panel).

all three have been isolated from natural ecosystems and/or human and animal hosts with ploidies that range from haploid to polyploid. Furthermore, all three species can undergo somatic ploidy changes under laboratory conditions.

S. cerevisiae

The budding yeast *S. cerevisiae* contains 16 haploid chromosomes and reproduces by mating and meiosis or asexually via budding. Environmental isolates of *S. cerevisiae* include haploids, diploids, and polyploids (12, 27–30). Clinical and industrial isolates also show a wide range of ploidies and aneuploidy (16, 29). For example, WGS of 145 *S. cerevisiae* clinical isolates found that 34% were triploid or tetraploid and 36% were aneuploid (16). Many of these ploidy changes are thought to be adaptive, but the correlation between environmental selection and ploidy changes is not well characterized (17).

C. neoformans

Naturally found in diverse environmental niches, *C. neoformans* is responsible for pulmonary infections as well as cryptococcal meningoencephalitis in immunocompromised individuals (31, 32). Basidiospores are thought to be the infectious propagules, which are inhaled into the lungs followed by dissemination into other organs (33). *C. neoformans* is normally found in the haploid state with 14 chromosomes and reproduces both sexually and asexually, and these haploid cells can vary in chromosome copy number due to nondisjunction events (34) or due to unisexual or bisexual reproduction (35). Dramatic ploidy changes have been observed during infection. Polyploid "titan" cells that range from 4N to >64N make up ~20% of the infectious population within the host tissue (36, 37). In conjunction with the ploidy increase, cell size, capsule structure, and cell wall structure are also modified. A haploid *C. neoformans* cell is generally 5 to 10 μm in diameter; titan cells can be much bigger, with some reaching upward of 50 to 100 μm in diameter (38, 39). Very little is known about the mechanism of titan cell formation; it may involve endoreplication due to alteration of cyclin proteins as is observed in *Drosophila melanogaster* and human hepatocytes (40, 41). Aneuploidy, in particular the amplification of chromosome 1, has also been observed in response to antifungal drug stress (42, 43).

C. albicans

The most common human fungal pathogen, *C. albicans*, contains eight homologous chromosome pairs. Though it was previously considered an obligate diploid organism (44, 45), alternative ploidy states have been described including haploid, triploid, and tetraploid cells (10, 15, 46–48). No meiosis has been observed in *C. albicans*. Rather, it undergoes a parasexual cycle in which cells of opposite mating type fuse to form tetraploids (49–53). Under nutrient starvation, tetraploid cells show an increase in genome instability that leads to the loss of individual chromosomes over time, returning progeny cells to either diploidy or (more often) near-diploidy (9, 50, 52, 54). Ploidy changes also occur in response to specific environmental conditions. For example, growth on alternative carbon sources (e.g., L-sorbose), exposure to antifungals (e.g., fluconazole), high temperature, and interactions with the host all result in ploidy changes and aneuploidy within a few cell divisions (15, 55–62). The molecular mechanisms driving these ploidy changes are currently unknown.

Other Fungi with Altered Ploidy Levels and Evidence of Aneuploidy

Aneuploidy has been found in many other fungi. *Ashbya gossypii*, a filamentous fungus, has a single syncytia with multiple nuclei, and each nucleus can have a different ploidy (63). In *A. gossypii*, ploidy increases with age, while stress exposure can shift a population with high ploidy variation toward a more homogenous, haploid population (63). Ploidy level variation and aneuploidy are also common in isolates of the amphibian chytrid pathogen, *Batrachochytrium dendrobatidis* (64), and in plant pathogens such as *Fusarium oxysporum* (65, 66). In fact, karyotype variability in *B. dendrobatidis* seems to be the norm rather than the exception; out of 22 isolates analyzed by WGS, 18 were aneuploid, with base ploidies ranging from diploid to tetraploid (64). While these are only a few examples of ploidy changes that are known to occur in the fungal kingdom, they highlight that ploidy changes play a central role in adaptive evolution and genomic diversity.

PLOIDY CHANGES IN THE CONTEXT OF LABORATORY MUTANTS: TOWARD A MECHANISM OF ASEXUAL PLOIDY CHANGE

The underlying mechanisms that drive ploidy changes are not completely understood (12). Some genes encoding ploidy regulators have been identified in yeast deletion mutant screens and gene overexpression studies (67). However, in many mutants the ploidy-altering phenotype is not 100% penetrant (see below), indicating

that there are redundant mechanisms that regulate genome copy number. Alternatively, the mutant genotype may stochastically acquire fitness-associated ploidy changes, in which case the ploidy change would be secondary to the initial gene mutation.

Here we will discuss mutations that affect cell cycle, spindle pole body (SPB), kinetochore attachment, cohesion, chromatin formation, and cytokinesis, focusing on their impact on ploidy changes. Many mutations that affect chromosomal instability can also cause ploidy increases or decreases. Importantly, because chromosome aneuploidy is frequently observed in mutants that undergo ploidy change, it is difficult to determine if aneuploidy itself is driving whole-genome ploidy changes. For example, a mutation may cause aneuploidy, which then gives rise to a whole-genome ploidy change. Alternatively, this mutation may first induce a whole-genome ploidy change, and subsequent aneuploidy results from increased genome instability. The examples provided below support that the mechanisms driving changes in whole-genome ploidy and chromosome copy number are extremely complex and often involve the essential machinery of the cell.

Mutations Underlying Ploidy Amplification

Alterations in cell cycle control can cause ploidy amplification. Endoreplication, the process in which DNA replication is followed not by cytokinesis, but instead by another round of DNA replication, has been described for multiple organisms (68–70). In the fission yeast *Schizosaccharomyces pombe*, cyclin B ($p53^{cdc\text{-}13}$) regulates the temporal order of DNA replication and mitosis. Control of cyclin B levels and the timing of this cell cycle regulator are important; formation of the $p34^{cdc\text{-}2}$-$p53^{cdc\text{-}13}$ complex specifies that the cell is in the G2 phase of the cell cycle. Loss of this complex redefines a cell in the G2 phase to a G1 phase cell, and the cells can then re-enter S-phase and rereplicate their genomes, causing unscheduled WGD. Re-entry into S-phase can occur multiple times and can lead to ploidy shifts from 1N to 32N (71). Interestingly, 42 uncharacterized genes were recently identified in human cells for their role in preventing endoreplication (72), supporting that DNA replication controls are still being discovered.

Under certain circumstances endoreplication is an environment-induced or programmed cell event. For example, environment-induced endoreplication likely leads to titan cell formation in *C. neoformans* (36, 38, 39). The exact mechanism of cell cycle alteration is not known for titan cell production, but like in *S. pombe*, the control of cyclin B level is a potential candidate. In the human liver, hepatocytes undergo genome replication followed by programmed cytokinesis failure to produce a binucleate daughter cell that is 4N (73). These binucleate tetraploids can then undergo another round of DNA replication followed by cytokinesis to generate mononucleate tetraploid cells. This cycle can continue to produce octaploid cells and so on. In addition, these polyploid hepatocytes can undergo mitosis with multipolar spindles, resulting in ploidy reduction and aneuploidy (8). It has been hypothesized that these aneuploid daughter cells may provide adaptive benefits during periods of cellular stress and allow for repopulation and restoration of the liver (73–75).

Accurate SPB function and attachment of the spindle microtubules to the chromosomes are required for proper chromosome segregation, and ploidy changes can occur when different components of the SPB complex are altered. During normal cell division, sister chromatids are attached to opposite SPBs and are pulled apart, thereby segregating the sister chromatids. *NDC1* (nuclear division cycle 1) encodes a subunit of the nuclear pore complex in *S. cerevisiae* and is required for SPB duplication and insertion into the nuclear membrane (76). Mutant *ndc1* cells have only a single functioning SPB to which all chromosomes attach and are then segregated into a single daughter cell, resulting in WGD.

Proper attachment of the SPB microtubules to the kinetochore is a major point of regulation during cell cycle progression. In *S. cerevisiae*, mutations in the essential gene *IPL1* (increased ploidy level 1) can result in aneuploidy and/or elevated ploidy level. *IPL1* encodes Aurora kinase, which is involved in the attachment of the spindle microtubules to the kinetochores, chromosome segregation, and checkpoints including mitotic spindle dis/assembly and DNA damage (77–79). Ipl1 is also responsible for sensing mitotic spindle attachment at the kinetochore and preventing segregation in cases where the chromosome is attached to only one SPB. Using a temperature-sensitive mutant of *IPL1*, Chan and Botstein (67) observed that haploid cells rapidly acquired multiple aneuploid chromosomes when grown at the restrictive temperature, and some of these mutants gained enough chromosomes to result in a ploidy increase.

In addition to SPB subunits, ploidy is affected by defects in chromosome cohesion. After replication, sister chromatids are packaged together by cohesion, a protein complex that holds the sister chromatids together until they are separated during anaphase (80). Improper loading or disassembly of the cohesion complex during the cell cycle leads to aberrant chromosome seg-

regation that results in ploidy shifts and aneuploidy (81). Defects in the cohesion complex, as well as regulators of the complex, lead to release of sister chromatids before proper attachment to the SPB. This increases the chance that sister chromatids will be inherited together because the sensing of microtubule attachment does not occur and segregation of sister chromatids is no longer interdependent (81).

Chromatin structure and regulation are important regulators of ploidy. The dynamics of chromatin structure are determined by histone conformation and modifications. Mutations within the globular domain of histone 4 (H4) result in heterogeneous colony sizes and an increased frequency of WGD and aneuploidy in *S. cerevisiae* (82). Further analysis of these colonies shows that small colonies consist of a mixed population of haploid and diploid cells, while large colonies are completely diploid, suggesting extensive autodiploidization. Alterations of the amino acids in the globular domain of H4 (L97, Y98, or G99 to alanine) do not alter the ability of H4 to interact with other histones. Instead, these mutations alter the H4 interaction with the histone chaperones Rtt106 and Caf-I. Disruption of these interactions prevents the nucleosome from being loaded onto the DNA. This decrease in histone occupancy could disrupt kinetochore architecture and assembly, leading to the increase in aberrant chromosome segregation and autodiploidization (82).

Just as histone modification can affect genome stability, nucleosome stability at centromeres is necessary for kinetochore assembly and proper chromosome segregation. Nucleosome stability is achieved through proper distribution of histone variants. The histone variant H2A.Z is conserved across all fungi and higher eukaryotes and is enriched at pericentric DNA but excluded from CENP-A (Cse4) nucleosome binding sites (83). Ies6 is an essential subunit of the INO80 chromatin-remodeling complex. Loss of Ies6 causes polyploidization and increased localization of pericentric H2A.Z (84), resulting in changes to chromatin structure that inhibit centromere/kinetochore function. Furthermore, overexpressing H2A.Z in an *ies6* mutant strain further increases chromosome instability, leading to more rapid polyploidization (84).

After genome replication and chromosome segregation, the cell undergoes a cleavage event controlled by the contraction of an actin ring found between the mother and daughter cell. Myo1, the sole myosin II motor in *S. cerevisiae*, associates with the actin ring and promotes cleavage between the mother and daughter cell (85). Haploid strains with a *myo1* deletion often undergo WGD events to survive, causing ploidy level increases from 1N to 4N (86). Many of the polyploid *myo1* evolved clones are mononucleate and contain multiple aneuploid chromosomes. These polyploid *myo1* evolved clones are not only viable but have restored cytokinesis through a variety of mechanisms, unlike most of the *myo1* cells that remain haploid. Therefore, the mutations that disrupt cell division and alter ploidy level can provide increased adaptability (86).

Mutations Underlying Ploidy Reduction

Several *S. cerevisiae* mutations have been identified that lead to genome reduction from diploid to haploid. For example, diploid cells with a null mutation in *RAD52* (involved in strand exchange during recombination and DNA damage repair) undergo chromosome loss via sequential aneuploid transitions (87). Loss of Rad52 leads to a failure of the repair mechanism that keeps the chromosome homologs together after a double strand break (DSB). Loss of this pathway gradually leads to genome reduction toward haploidy over ~500 generations. Interestingly, cells with higher ploidy showed increased Rad52 dependency because *RAD52* is essential for growth of tetraploid cells but not isogenic diploid cells (88). This supports that increasing ploidy results in an increased frequency of DSBs that must be repaired by Rad52, and diploids, but not tetraploids, can resolve some of this damage by sequential chromosome loss and eventual ploidy reduction.

In addition to mutations in *RAD52*, null mutations in *CTF18* (involved in sister chromatid cohesion) also lead to rapid genome reduction from diploidy to haploidy (89). This reduction in ploidy occurs over a relatively short amount of time (~50 generations) and suggests that the haploid cells have a fitness advantage over the diploid progenitor. Surprisingly, genome duplications are also observed within the diploid *ctf18* population, resulting in a heterogeneous population (haploid, diploid, aneuploid, and polyploid cells). This suggests that specific mechanisms of genome instability may simultaneously induce aneuploidy, ploidy loss, and ploidy gain.

IMPACT OF PLOIDY LEVEL ON DNA DAMAGE REPAIR

At all ploidy levels, DSBs are repaired by two main pathways: nonhomologous end-joining and homologous recombination. Nonhomologous end-joining repairs DSBs via ligation of the broken DNA ends with little or no processing of the DNA ends. Nonhomologous end-joining is considered error-prone because this process can introduce novel mutations. Alternatively,

homologous recombination uses a homologous DNA sequence to serve as a donor for recombination and DSB repair (90, 91). The availability of homologous DNA sequences (sister chromatid, homologous chromosome, or an ectopic sequence) is influenced by the ploidy of the cell and phase of the cell cycle: haploid cells have homologous DNA sequences available during S/G2 only, while diploid and polyploid cells have homologous DNA sequences available throughout the cell cycle. Therefore, diploid and polyploid cells have an increased preference for homologous recombination to repair DSBs, resulting in increased frequencies of recombination, gene conversion, crossover events, and gross chromosomal rearrangements (GCR) (92–94).

Isogenic yeast strains with different ploidies exhibit different mutation rates and sensitivity to DSBs. The forward mutation rate at either the *CAN1* or *URA3* locus is two orders of magnitude higher in diploids than in haploids, and diploids are less sensitive to DSBs than haploids (88, 89, 95). Interestingly, the forward mutation rate at *CAN1* is lower in tetraploids compared to diploids (88). Ploidy also affects sensitivity to DSBs: both haploids and tetraploids are more sensitive to DSBs than diploids. One reason for these observations may be the different requirements for DSB repair at different ploidy levels (94).

Ploidy-specific genome maintenance mechanisms may exist, but many questions still remain (94). For example, in *S. cerevisiae* there are ploidy-specific stress responses to GCR (96), and tetraploid yeasts have a greater requirement for genes involved in recombination and mitosis than haploids or diploids (88). This phenomenon, called "ploidy-specific lethality," is used to identify the physiological alterations that accompany tetraploidy. Out of 3,740 yeast deletion mutants, only 39 genes exhibit ploidy-specific lethality. Almost all of these mutations affect genomic stability by impairing homologous recombination, sister chromatid cohesion, or mitotic spindle function (88). Why tetraploid cells have an increased requirement for these genes remains unknown (97). The rate and spectrum of mutations available to each ploidy level is remarkably different, so genome maintenance mechanisms could play an important role during adaptation.

GENOME INSTABILITY IN POLYPLOID CELLS

One of the most striking features of polyploid cells is their increased genome instability relative to diploid cells. This phenomenon is seen across many eukaryotic species, including many plant species (98–101). In fungi, polyploid cells frequently give rise to aneuploid progeny (both whole chromosome and segmental), chromosome rearrangements, and translocations. For example, tetraploid *S. cerevisiae* cells have a 200- to 1,000-fold increase in the rate of chromosome loss compared to isogenic diploid cells (88, 102, 103). Similarly, tetraploid *C. albicans* cells have a 50-fold higher rate of loss of heterozygosity (LOH) compared to isogenic diploid cells (54). Increased genome instability often results in rapid ploidy reduction during *in vitro* growth of most polyploid populations (9, 19, 20, 43, 54, 104), yielding progeny with high karyotypic diversity. Here we highlight examples for *C. neoformans*, *C. albicans*, and *S. cerevisiae*.

The dramatic ploidy reduction of *C. neoformans* titan cells was recently analyzed by microdissection of sequential daughter cells from a polyploid titan cell followed by colony formation assays and WGS (43). In a replete environment, polyploid titan cells produced true haploid daughter cells. In contrast, when exposed to fluconazole, titan cells frequently generated aneuploid haploid and aneuploid diploid daughter cells (43). Furthermore, daughter cells from the same titan cell parent had diverse aneuploid karyotypes, while point mutations were rarely observed. It remains to be tested whether this aneuploidy is due to an effect of fluconazole on mitotic fidelity (as was seen in *C. albicans* [62]) or is the result of a general stress response.

A foundational study of *C. albicans* tetraploid cells found that they undergo a rapid, nonmeiotic genome reduction in response to nutrient starvation, termed "concerted chromosome loss" (9). Genetic analysis of many progeny by microarray (single nucleotide polymorphism [SNP]) and aCGH arrays) found that only a very small fraction returned to a true euploid diploid state, and the majority of the progeny were aneuploid for one or more chromosomes. In addition, recombination, albeit limited to a few progeny, occurred between heterozygous loci on homologous chromosomes during tetraploid genome reduction (52). The appearance of multiple gene conversion tracts within several strains, and the general absence of gene conversion tracts in other strains, suggests that some cells become generally competent for recombination at more than one locus, while other strains do not undergo such recombination events at all. Importantly, these recombination events were dependent on Spo11, a conserved protein required for the introduction of DSBs (52). These findings suggest that at least one meiosis-specific gene has been reprogrammed to mediate genetic recombination during the alternative parasexual life cycle of *C. albicans*.

Recent studies have characterized the rate of concerted chromosome loss in different growth environments. For example, populations of tetraploid, triploid, and diploid *C. albicans* strains were grown for 28 days in yeast extract-peptone-dextrose (YPD), and chromosome loss dynamics were analyzed at different time points using flow cytometry and ddRADSeq. Initially, the tetraploid-evolved clones were highly aneuploid, and once the cells became aneuploid, further ploidy reduction accelerated, with aneuploid strains changing ploidy faster than tetraploid strains (54). Eventually, the tetraploid and highly aneuploid evolved cells converged to stable euploid levels: diploid, triploid, and (rarely) tetraploid. In addition, this study showed that chromosome loss was random; ploidy reduction in biological replicates of the same polyploid strain rarely followed the same chromosome loss trajectory.

Similar nonmeiotic ploidy reductions have been observed in tetraploid populations of *S. cerevisiae* during *in vitro* evolution (19). In this study, tetraploid *S. cerevisiae* strains were passaged for ~1,800 generations in rich or high-salt medium, and genome reduction at the population level was detected with flow cytometry after ~186 generations. Results from this study support that ploidy loss occurs as if selection is acting on multiple chromosome loss events simultaneously rather than on individual, sequential chromosome loss events (105). In addition, these *in vitro* evolution experiments found that ploidy loss in a tetraploid population often paused at near-euploid levels, from tetraploid to near-triploid to near-diploid (105). Furthermore, aneuploidies (detected by aCGH) present in the tetraploid progenitor were often found to be the only aneuploidy that remained in the near-diploid evolved population (19). Thus, while the mechanism of tetraploid genome reduction is unknown, the rapid ploidy loss observed in these environments supports that multiple chromosomes can be lost simultaneously.

The ploidy reduction process of *S. cerevisiae* tetraploid cells was further analyzed with WGS of strains from many parallel *in vitro* evolution experiments (20). The ploidy of tetraploid-evolved clones was determined by flow cytometry, and the copy number of every chromosome was determined by WGS. It was found that aneuploidy is more extensive during the tetraploid to diploid transition than previously appreciated. In raffinose medium, ploidy reduction did not occur by loss of a full set of chromosomes; rather, many of the tetraploid evolved cells were highly aneuploid, including individual chromosome copy numbers that ranged from 4C to 2C in the same cell (Fig. 2). This indicates that while some chromosomes are lost multiple times, others are maintained at the original tetrasomic level (e.g., 4C, 3C, and 2C). Interestingly, specific chromosome pairs were more frequently lost together and underwent multiple loss events independent of other chromosome pairs (20). These copy number differences were likely a result of the environmental selection for particular karyotypes with increased fitness; indeed, the most common aneuploid chromosome in this study, amplification of chromosome XIII, was found to provide a significant fitness benefit to tetraploid cells in raffinose medium (20).

These studies of *C. albicans* and *S. cerevisiae* show that tetraploid-evolved clones can be highly aneuploid, with many evolved isolates reaching a near-euploid state (near-triploid or near-diploid). The rate of chromosome loss is largely dependent on the growth environment and generation time, as well as the frequency of sampling throughout the experiment. In general, the random chromosome loss that occurs during *in vitro* culturing of tetraploid *C. albicans* and *S. cerevisiae* populations appears mechanistically distinct from the haploid bud produced from *C. neoformans* titan cells. Future studies in these and other fungi may identify unique mechanisms of chromosome and genome stability and aneuploid tolerance in polyploid cells.

Given that ploidy reductions can occur over short evolutionary timescales, it is puzzling that polyploid cells are observed at all. One hypothesis is that polyploidy is simply an intermediate state to which a cell shifts for survival, for example, after cytokinesis failure due to cell wall stress (86, 106). However, another hypothesis is that the polyploid state is beneficial in specific environments and is selected upon due to increased fitness. Indeed, tetraploid *S. cerevisiae* strains passaged *in vitro* for ~1,000 generations at 23°C stably maintain euploid tetraploid genomes (107). These evolved tetraploid cells are resistant to benomyl, a microtubule depolymerizing drug that tetraploid cells are more sensitive to than diploids (88). The stable tetraploids have increased levels of Sch9, a kinase involved in protein homeostasis, G1 cell cycle progression, nutrient signaling, and stress response, suggesting that newly formed tetraploid cells can acquire genome stability through improved cell growth processes (107).

IMPACT OF PLOIDY ON FUNGAL PHYSIOLOGY

Alterations in genome architecture have wide-reaching effects on many cellular processes. How ploidy shifts change cellular physiology is a long-standing question. A ploidy increase is associated with enlarged cell size in

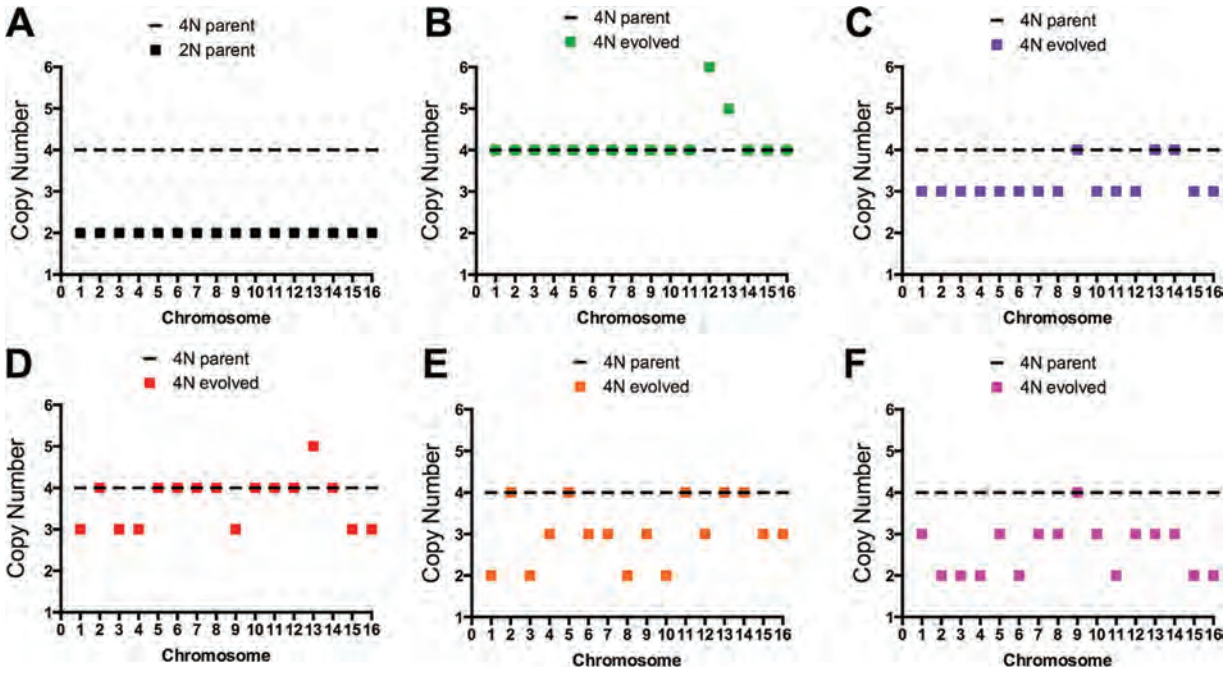


Figure 2 Many polyploid-evolved clones are highly aneuploid. Chromosome copy number was determined by WGS and plotted for the (**A**) parental diploid (2N) and tetraploid (4N) strains and different tetraploid evolved clones after 250 generations in raffinose medium. Adaptation resulted in clones with (**B**) increased chromosome copies, (**C**) approximately trisomic copies of every chromosome (~3N), or (**D-F**) highly aneuploid genomes. Figures generated from data obtained from the supplementary data Table 1 in reference 20.

all eukaryotes (38, 39, 108–111). However, we lack a mechanistic understanding of how genome size controls cell size (112). Research that has compared growth rate, gene expression patterns, and cell surface area to volume ratios between haploid and diploid, or diploid and polyploid cells has provided insight into the complex, sometimes subtle, differences between isogenic fungal strains of different ploidy. Here we highlight how ploidy affects the physiological properties of cell size and gene expression.

The reduction in cell surface area to volume ratio of polyploid cells likely impacts nutrient transport, signaling pathway components, and ultimately growth rate in some environments. Upon genome duplication, polyploid cells underwent a 2-fold increase in cell volume, but only a 1.57-fold increase in cell surface area relative to their diploid progenitors (88, 113, 114). This reduced surface area in polyploid cells was expected to cause growth disadvantages in environments where nutrients are absorbed across the membrane. However, there was little evidence to support this cell surface area-to-volume ratio theory in polyploid cells (115). A robust analysis of this theory, performed with 51 haploid and diploid *S. cerevisiae* and *S. paradoxus* strains in 33 growth conditions, found that ploidy accounts for a majority (70%) of the differences in growth rate between strains (17). However, the results were inconsistent with the surface area-to-volume model. Rather, it appeared that the preference for the haploid versus diploid state was due to condition-specific effects in the strains tested, not due to differences in surface area (17). Thus, more research is needed to understand the physiological changes that occur after polyploidization and what effect the environment has on fitness and genome stability. Furthermore, identifying transcriptional changes that occur in other environments, including nutrient limitation, will provide important clues to the growth differences polyploids experience in these environments.

Gene expression studies have been used to identify molecular signatures that are unique to polyploid cells. The first gene expression microarray study on polyploid *S. cerevisiae* cells identified only a few genes with significantly different levels of expression in isogenic haploid and tetraploid cells when grown in rich medium (114). For example, tetraploid cells had reduced expression of genes encoding G_1 cyclins (*CLN1* and *PCL1*) and proteins involved in cytoskeletal orga-

nization (e.g., *GIC2*). In another study, a different *S. cerevisiae* strain (S288C instead of Σ1278b) was analyzed under the same growth conditions, and no genes had significantly different levels of expression in isogenic diploid and tetraploid cells (88). This suggests that either there are more transcriptional differences between haploids and tetraploids compared to diploids and tetraploids, or there are strain-specific ploidy differences (116).

Using RNA sequencing analysis, Wu et al. identified 65 transcripts with differential expression in isogenic haploid and tetraploid *S. cerevisiae* cells, again in rich medium (109). A majority of these genes encoded cell surface proteins, which suggests that the enlarged cell size of tetraploids caused the differential regulation of these genes, and not ploidy *per se*. Indeed, it was found that haploid mutants with increased cell size showed differential expression for many of the same genes as tetraploids, and the degree of changes in expression correlated with the degree of size increase (109). Importantly, gene expression of most of these cell surface proteins was repressed in tetraploid cells (and enlarged haploids), likely due to the reduced surface area relative to cell volume (88, 113, 114). These observations are consistent with proteomic studies that found reduced amounts of cell surface proteins in diploids compared to haploids (117).

PLOIDY CHANGES AND ANEUPLOIDY IN THE CONTEXT OF EXPERIMENTAL EVOLUTION

Ploidy Changes and Aneuploidy Occur During *In Vitro* Evolution and Provide a Fitness Benefit

In vitro evolution experiments provide the strongest evidence that fungi can undergo asexual ploidy changes during adaptive evolution. The environmental cues driving these genome changes are not known, and a comprehensive study of environments that induce ploidy changes (WGD and ploidy reduction) is lacking (Table 1). In this section, we will examine the different environments in which ploidy changes and acquisition of aneuploidy are documented in fungi and how these genome size changes affect organismal fitness and adaptation.

We describe the *in vitro* studies in which whole-genome ploidy changes and chromosome copy number changes are observed. Many of these examples describe changes in ploidy that occur at the population level. If beneficial (e.g., increasing fitness), ploidy or chromosome copy number changes are selected for within the population. The exact order of events and mechanisms that drive the initial ploidy change is unknown.

Results from multiple studies showed that in *S. cerevisiae*, whole ploidy level changes causing diploidization are advantageous in multiple environments. For example, isogenic haploid, diploid, and tetraploid *S. cerevisiae* populations passaged in rich medium, medium containing high salt, or increased concentrations of ethanol all converged toward diploidy (19, 105, 118). The diploidized cells had significantly higher fitness than the isogenic progenitor strains in competition experiments (118). Furthermore, the rate of ploidy change is dependent on the specific growth conditions. For example, in high-salt medium, the rate of diploidization of haploids was faster than the rate of diploidization of isogenic tetraploids (19). Therefore, becoming diploid under these growth conditions was one of the most common and accessible routes to increased fitness.

Frequent diploidization of haploid cells also occurred during *in vitro* evolution experiments under glucose depletion (21, 119). In this unique study, the evolving populations consisted of ~500,000 uniquely barcoded haploid *S. cerevisiae* lineages. The autodiploidized clones had a strong fitness benefit relative to their haploid progenitor, and WGS revealed that in most cases diploidy was the only adaptive mutation (21). The autodiploid lineages were traced back to their respective founding populations, suggesting that the transformation conditions (lithium acetate and heat shock) used for barcoding led to the formation of autodiploids (21). Interestingly, transformation conditions also induced ploidy loss, aneuploidy, and LOH in *C. albicans* (55, 120).

Convergence toward diploidy is also observed in *C. albicans*. Tetraploid mating products and rare haploid isolates returned to the diploid state after *in vitro* passage (9, 47, 54). The convergence toward diploidy occurred under different growth conditions but ultimately depended on the environment and initial genotype, not necessarily the fitness benefit of the acquired ploidy change (121). For example, during phosphorus depletion, the autodiploidization rate of haploids was reduced, while the diploidization rate of tetraploids was increased. Further studies are needed to uncover the factors that drive organisms to a "baseline" ploidy.

In contrast to the convergence toward diploidy observed in many environments, a wide array of genome changes can occur under a single growth condition. For example, growth in the fungistatic drug fluconazole often resulted in a wide variety of ploidies and aneu-

Table 1 Summary of experimental evolution studies in fungi and the ploidy and aneuploidy associated with different environmental stresses. Ploidy levels of haploid (1N), diploid (2N), triploid (3N), and tetraploid (4N) are euploid states, while aneuploidy is indicated if known.

Fungi	Stress	Timeframe	Ploidy change/aneuploidy	Reference(s)
S. cerevisiae	YPD, high salt	1,800 generations	1N → 2N and 4N → 2N	19
S. cerevisiae	YPD	186 generations	4N → 2N and 3N → 2N	105
S. cerevisiae	Raffinose	250 generations	4N → 4N–2N aneuploidies common 2N → 2N and 1N → 1N (no ploidy change)	20
S. cerevisiae	Ethanol	200 generations	1N → 2N and 4N → 2N	118
S. cerevisiae	Low glucose (LiAc transformation)	88–132 generations	1N → 2N	21, 119
C. albicans	L-Sorbose, presporulation at 37°C	5–7 days	4N → 2N and aneuploid 2N	9, 52
C. albicans	L-Sorbose	2 days	2N → 2N monosomic for Chr5	56
C. albicans	YPD	28 days	4N → 4N, 3N, or 2N 2N → 2N	54
C. albicans	Rich media and nitrogen depletion	140 generations	1N → 2N and 4N → 2N	121
C. albicans	Minimal media and phosphate depletion	140 generations	1N → 1N and 4N → 2N	121
C. albicans	Fluconazole (2–10 μg/ml)	8–12 h	2N → 4N, 8N, 16N trimeras (20% of cells in the population)	62
C. albicans	Fluconazole (100 mg disk)	12–16 h	2N → 1N	123
C. albicans	Fluconazole (increasing MIC)	330 generations	2N → 2N–3N aneuploidies common: whole chromosome, isochromosomes, and telomere-telomere fusions	46, 59, 61
C. albicans	Murine host	1–5 days	2N → 1N and aneuploid 1N	47
C. albicans	Murine host	Single passage	2N → 2N aneuploid	60
C. albicans	Heat shock (51°C)	90 seconds	4N → 2N	55
C. tropicalis	L-Sorbose	8–10 days	4N → 2N	104
C. tropicalis	Rich media	>120 generations	4N → 2N	104
C. neoformans	Fluconazole (32 μg/ml)	3–5 days	1N → 1N aneuploidies common	42, 124
C. neoformans	Fluconazole (8 μg/ml)	48–96 h	Polyploid titan cells → 1N and 2N aneuploidies common	43
C. neoformans	YPD, oxidative and nitrosative stress	48 h	Polyploid titan cells → 1N	43
C. neoformans	Murine	3 days	1N → polyploid titan cells (20% of population)	38
C. neoformans	Unisexual reproduction	~2 weeks	1N → 2N and aneuploidies common	35
A. gossypii	NaCl, $ZnSO_4$, fluconazole (325 nM), heat (37°C)	6 h	Polyploid and aneuploid → euploid 1N (distribution of ploidies shifted toward haploid)	63
A. gossypii	Caffeine	6 h	Polyploid and aneuploid → polyploid and aneuploid	63

ploid changes in *C. albicans* and *C. neoformans* (35, 43, 46, 59, 61, 62, 122–124), and just 8 to 12 hours of drug exposure resulted in both ploidy reduction and ploidy amplification in *C. albicans* (62, 123). Fluconazole is known to alter membrane fluidity, which causes abnormal cytokinesis and cell cycle defects leading to polyploid and aneuploid formation (62). The immediate fitness effect of these ploidy level mutations is not known, but it can be inferred by measuring how rapidly mutant cells accumulate within a population. For example, during *in vitro* evolution of *C. albicans* in the presence of fluconazole, acquisition of multiple aneuploid chromosomes was detected after just ~3.3 generations in the majority of cells in the population (61). One of these aneuploidies, isochromosome 5L [i(5L)], conferred fluconazole resistance due to the amplification of two genes, *ERG11* and *TAC1* (13, 59). Due to the high fitness benefit, i(5L) accumulated rapidly in the population, and in many cases was maintained over ~330 generations in the presence of fluconazole (61).

Many additional examples support that acquisition of aneuploid chromosomes can lead to rapid adaptation to specific environments, and like ploidy changes, these aneuploid chromosomes are reversible. For example, growth on alternative carbon sources led to loss

of specific chromosomes in multiple fungal species, and the addition of glucose led to reduplication of the remaining chromosome (56, 104). Additionally, during adaptation to heat stress, *S. cerevisiae* rapidly duplicated chromosome III, but after continued heat stress the chromosome aneuploidy was lost while elevated expression of genes on this chromosome was maintained, suggesting that aneuploidy provided time for cells to search for optimal adaptive solutions (125).

While acquisition of aneuploidy is not always beneficial (126), aneuploidy can lead to a wide variety of fitness effects in different growth environments. Studies of the heat shock protein Hsp90 in *S. cerevisiae* showed that during periods of heat stress Hsp90 was titrated away from its regular clients such as kinetochore proteins (127, 128). Importantly, exposure to heat stress or inhibition of Hsp90 led to a marked increase in chromosome instability and tolerance to other stressors including hydrogen peroxide, cyclohexamide, tunicamycin, benomyl, and radicicol (128). More recently, Dunham and colleagues performed a comprehensive analysis of aneuploid genotypes in *S. cerevisiae* and found that each karyotype had large, but condition-dependent, changes in fitness, supporting that aneuploidy can be an important driver of adaptation (129, 130). Many more *in vitro* evolution experiments showed that aneuploidy and ploidy change can be beneficial during adaptation, especially during adaptation to nutrient limitation and heat stress (55, 120, 131).

While we have discussed how changes in ploidy and chromosome copy number can be adaptive in certain environments, some species of pathogenic fungi carry multiple, nonessential chromosomes called accessory, supernumerary, B, or dispensable chromosomes (132). Accessory chromosomes are characterized by highly variable regions, high mutation rates, and genes acquired via horizontal gene transfer (133–136). Many of these harbor virulence genes that allow the fungus to infect its host. The plant pathogen *Nectria haematococca* carries an array of genes on accessory chromosome 14 that allow the fungus to detoxify phytoalexin used by the pea plants as a defense mechanism (136, 137). In *F. oxysporum*, a striking 40% of the genome was found exclusively in strains that infect tomato plants. Moreover, most of the genetic material in these accessory chromosomes lacks sequence homology to any close relatives of *F. oxysporum* (138). Further research is needed to investigate how these accessory chromosomes evolved, are maintained, and what roles they play in virulence.

Many filamentous fungi, including the cotton pathogen *A. gossypii*, have evolved alternative strategies to generate genotypic diversity. This fungus has syncytia that undergo asynchronous nuclear division (139). It was originally thought to be a predominantly haploid fungus, but Gladfelter and colleagues recently demonstrated frequent ploidy variations for different nuclei within the same syncytium, including aneuploidy (63). To identify ploidy changes, they used lac operator arrays to track the copy number of individual chromosomes within the nuclei of the same syncytium and found nuclear ploidies ranging from 1N to >4N (63). Interestingly, in response to cellular stress (cell wall stress, osmotic stress, excess zinc, the antifungal fluconazole, and increased temperature), polyploid nuclei diminished and haploid nuclei predominated. These results suggest that nuclei with different ploidies are tolerated within single syncytia, and that there may be costs associated with ploidy level variation, because stress homogenizes the genome content of nuclei (63). Remarkably, ploidy reduction and homogenization of ploidy under stress is the opposite of what has been observed for the human fungal pathogens *C. albicans* and *C. neoformans*, where stress led to genomic diversification within populations and increased aneuploidy (43, 59, 61).

The above examples establish that fungal organisms can adapt rapidly to their environment by undergoing ploidy and chromosome copy number changes. It also highlights the challenge of determining the causative interactions between environment and ploidy levels (Table 1). While it is unclear what types of ploidy changes will arise in any given environment, it is a common strategy of pathogenic fungi to generate genomic variation in response to environmental perturbations.

It is important to note that in any given fungal population, one or more subpopulations may exist that have acquired viable aneuploidies or undergone ploidy shifts. This is due to background rates of chromosome mis-segregation and failed mitosis. It is of great interest to understand how entire populations of cells, when subjected to stress, can become polyploid or aneuploid. Are the vast majority of cells dying and selection acts upon the survivors, resulting in cells with ploidy changes beneficial for survival? Or could there be a mechanism permissive or instructive in the generation of aneuploid or polyploid organisms? Both of these questions remain open to further investigation.

Ploidy and Aneuploidy Changes Arise During *In Vivo* Evolution

Ploidy level heterogeneity and aneuploidy are frequently observed in clinical isolates of fungal species including *C. albicans*, *C. neoformans*, and *Candida glabrata*

(10, 11, 15, 140, 141). However, very little is known about the dynamics of ploidy changes during *in vivo* evolution and the effects on host-fungus interactions.

To assess the types of genome alterations that *C. albicans* undergoes upon host encounter, single-passage *in vivo* experiments were conducted in a murine model of systemic infection. Ploidy changes compared to the diploid progenitor were detected in ~3% of recovered strains and included segmental and whole chromosome aneuploidy. While this frequency is relatively low, no aneuploids were detected in strains from an *in vitro* control population. Results from this study suggest that conditions within the animal host affect chromosome disjunction more strongly and that this may reflect the very different population growth responses *in vivo* (60). More recent studies on the adaptive potential of *C. albicans* in response to the oral and systemic host niche show that the acquisition of aneuploidy is much more frequent than previously reported. Excitingly, the first haploids ever reported for *C. albicans* were found among strains recovered from an oral model of infection (47). Haploid isolates arose as early as 3 days postinfection with a frequency of $\sim 2 \times 10^{-5}$ and not only differed in their genotypes (e.g., *MTL* status) but also showed phenotypic differences compared to their diploid progenitor. These phenotypes include decreased fitness in YPD at 30 and 37°C and defects in filamentation (47; A. Forche, unpublished). Importantly, haploids arose independently in six mouse hosts, and multiple haploids with different genotypes were found in the same host (A. Forche, unpublished). Overall, the frequency of phenotypic changes such as fitness at different temperatures, filamentation, or the production of hydrolytic enzymes that accompanied aneuploidy in general was much higher compared to cells that remained diploid. This suggests that some of these aneuploid states may be adaptive. Interestingly, the dramatic changes in ploidy may be specific to pathogenic host niches: passage of *C. albicans* using a murine commensal model found no ploidy or chromosome copy number changes by flow cytometry and WGS (142), supporting that host microenvironment impacts genome architecture.

Upon infection with *C. neoformans*, the host's immune system responds by trying to combat the fungus via oxidative or nitrosative attacks by macrophages and neutrophils. The formation of polyploid titan cells inside the host (as discussed above) is accompanied by a ploidy shift from a 1N euploid state to one up to >64N (143). Current evidence points to the importance of titan cells and their ability to withstand various stresses that may be encountered within a host (38, 43). For example, among cells harvested from infected lungs, titan cells exhibited resistance to both sodium nitrate and *tert*-butyl hydroperoxide and survived under conditions lethal to non-titan cells (38, 43).

In summary, single passage of diploid *C. albicans* cells in an oropharyngeal model of infection leads to ploidy reduction to a haploid or near-haploid state (47). In addition, aneuploidy arises frequently upon encounter with the host in different models of infection (60). In contrast, haploid *C. neoformans* undergoes polyploidization upon host encounter (144). It is currently unknown what specific factors influence ploidy shifts in these fungi, but it is likely a combination of many variables including temperature, pH, host immune cells, and interaction/competition with other microbes (145, 146).

THE BENEFICIAL EFFECTS OF PLOIDY CHANGES

Adaptation of an organism to a novel environment is a function of the rate in which beneficial, growth-promoting mutations are acquired and spread throughout the population. The rate of adaptation is affected by multiple, interrelated properties that determine the appearance, persistence, and fixation of beneficial mutations in a population. These properties include the rate of beneficial mutations, the fitness effect of these mutations, the dominance of mutant alleles, and effective population size (147–153). While aspects of this process are still poorly understood, theoretical studies indicate that ploidy affects the rate of adaptation (2, 154, 155).

Ploidy change can impact the evolutionary trajectory of a cell by altering the rate and spectrum of beneficial mutations. For example, a ploidy increase (WGD) increases the mutational target size. A ploidy increase also provides the cell with the ability to buffer deleterious mutations due to redundant gene copy numbers (1, 156). Furthermore, some mutations and/or genome changes are only accessible to cells with specific ploidies, and this can have a profound impact on adaptation. For example, diploid and polyploid cells can undergo LOH, while haploid cells can acquire recessive mutations upon which selection can act (1, 157, 158). Similarly, polyploid cells have high rates of whole-chromosome aneuploidy, which can provide fitness benefits during adaptation (20, 86, 88), yet haploid cells rarely acquire aneuploidy during adaptation, likely due to the increased fitness cost of these mutations in most environments (126). Lastly, the fitness effect of a given mutation is assumed to be equal across all ploidy levels, but recent

experimental evidence suggests that this is not the case for all mutations (20, 159–161).

IMPACT OF PLOIDY AND ANEUPLOIDY ON CANCER BIOLOGY

Ploidy changes and acquisition of aneuploid chromosome copy numbers are hallmarks of cancer cells. Tetraploid cells are frequently observed in precancerous lesions of Barrett's esophagus (162, 163) and in preneoplastic cervical cells (164). Recently, a systematic comparison of over 5,000 human tumors found that 37% had undergone genome duplication during tumorigenesis (165). Evidence for ploidy changes in these tumors came from gene copy number and allelic ratio data obtained from SNP microarrays. Compared to diploid tumors, tumors that underwent WGD had higher rates of gene copy number alterations, including chromosome loss, resulting in ploidy levels ranging from triploid to tetraploid. WGD often preceded other gene copy number alterations in these tumors, supporting that WGD can be a driver of tumorigenesis. The idea that WGD can promote tumor development was previously shown in a mouse breast cancer model (p53–/– mammary epithelial cells [166]). Isogenic diploid and tetraploid cells were generated *in vitro*, mimicking a recent WGD event. When transplanted into mouse mammary epithelia, only the tetraploid cells generated tumors (166). Additional studies support that WGD resulting in tetraploidy is an intermediate step toward aneuploidy and tumorigenesis (106, 164), while other studies support that in some cancer types aneuploidy may precede the tetraploidization event (167).

Whole-genome reduction is also observed during development of rare leukemia subtypes, including acute lymphoblastic leukemia (168). These haploid leukemias are considered near-haploid because the lymphoblastic cells are often aneuploid for one or more chromosomes (1N+1), frequently involving chromosome 21 (168). Haploidization and LOH nearly across the entire genome may underlie the aggressiveness of these cancers, resulting in very poor prognosis (168). However, this haploid population appears to be unstable and undergoes WGD (autodiploidization) with some frequency. Evidence for autodiploidization was found in patients with mixed lymphoblast populations of both a near-haploid karyotype (1N+1) and a near-diploid population that was exactly twice that of the aneuploid karyotype (2N+2) (168). These data support that ploidy variation is directly linked to cancer evolution, yet the effects of ploidy change versus the effects of aneuploidy *per se* are difficult to distinguish.

CONCLUDING REMARKS AND OUTLOOK

There is a common theme in the examples discussed in this review: somatic ploidy changes increase the genetic heterogeneity of a population of cells (169–171). This genetic heterogeneity includes increased frequency of aneuploidy, but importantly, both ploidy and aneuploidy are reversible genomic changes. For example, a diploid cell that has undergone chromosome loss can later duplicate the remaining homologue (albeit losing heterozygous information on the lost chromosome). Similarly, WGD from a diploid to a tetraploid cell frequently results in chromosome loss and eventually ploidy is returned to the diploid state.

The initial ploidy-changing event may arise very rarely in some environments, but if there is a fitness benefit that accompanies the ploidy change, then it will spread throughout the population over time. What is surprising then is how often these ploidy-changing events occur within short-term *in vitro* evolution experiments and over eons of evolution. Perhaps the adaptable genome is the most successful genome, and cells with the ability to undergo rapid genome expansion and/or contraction are able to survive the most environmental insults. Additionally, it is possible that there is a subpopulation of persister cells that are primed and/or programmed to undergo ploidy changes during the response to stress. More population-level and single-cell approaches are needed to define the mechanisms and environmental conditions that cause ploidy changes in all eukaryotes (especially during fungal infections and tumorigenesis). The impressive examples of genomic heterogeneity observed in fungal pathogens support using these simple model systems to determine the mechanisms of ploidy change that occur during the somatic evolution of human cancers.

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Fungal Genomes and Insights into the Evolution of the Kingdom

29

Jason E. Stajich

EVOLUTIONARY RELATIONSHIPS OF FUNGI

Studies of fungal evolution require an understanding of the phylogenetic relationships and relative evolutionary divergence of organisms. The first approaches to organizing fungi into related groups relied on morphological characteristics (1). These approaches provided a broad framework to organize fungal organisms for taxonomic classification based on recognizable morphological characteristics such as spore shape, asexual and sexual structures, and in mushroom-forming fungi, the shape and presence/absence of gills, veil attachments, and spore color. In zoosporic chytrid fungi the characteristics seen by scanning electron microscopy of zoospores reveal that the ultrastructure of the kinetosomes and flagellum are all diagnostic for the classification of many lineages (2). However, the microscopic nature of many fungi and especially of yeast-forming fungi with limited visible differences, and the prevalence of convergent evolution to homoplasies or similar characteristics across a tree, has made taxonomic classification of groups of fungi difficult or easily misled. The invention and application of DNA sequencing (3) and PCR (4) and the development of primers to amplify fungal rRNA enabled a new era of molecular phylogenetic studies in fungi (5). These approaches provided invaluable information that was used to resolve the major fungal lineages (2, 6–20) and the delineation of species (21–24). Using DNA approaches to study the entire fungal tree of life provided new insight into the order of branching of major groups and the timing of morphological changes such as the loss of the flagellum found in zoosporic fungi (14, 17, 18, 25).

With improved resolution of the phylogeny of fungal species, the timing of the emergence and changes in species traits and lifestyles can be compared, such as the evolution of host-associated symbioses and pathogenic interactions with plants or animals. Analysis of the evolution of morphological complexity along a phylogeny can establish the approximate time when complex growth emerged, such as fruiting bodies and multicellular structures, or simplified to establish transitions when unicellular yeast morphology emerged (26). Changes in subcellular characteristics such as septate hyphae, polarized hyphal growth, and the presence or absence of a flagellated life stage can be mapped on the tree to study the order and timing of their emergence relative to other structures or host-associated lifestyles (27, 28). With the availability of genome sequence data, the history of these phenotypic changes can be mapped onto the phylogeny and compared to the evolution of individual genes or the presence or absence of a gene family or be correlated with changes in evolutionary rates of gene sequences (29). These changes may reflect gains, but losses can also drive morphological changes such as simplification (26). The data and approaches are only recently becoming available to support robust analyses to link genes to phenotypes. Some examples of successful applications of the approaches include the acquisition of enzymes needed for anaerobic growth by Saccharomyces (30–32), gains of subtilase genes related to animal-associated lifestyles (33–35), genome reduction correlated to a yeast growth form (36, 37), and changes in structure of centrioles for microtubule attachments (38).

As technology advanced to support inexpensive high-throughput genome sequencing, improved phylogenetic software and high-performance computing have further improved the resolution of the phylogenetic relationships of fungi (Fig. 1). Fungi were some of the

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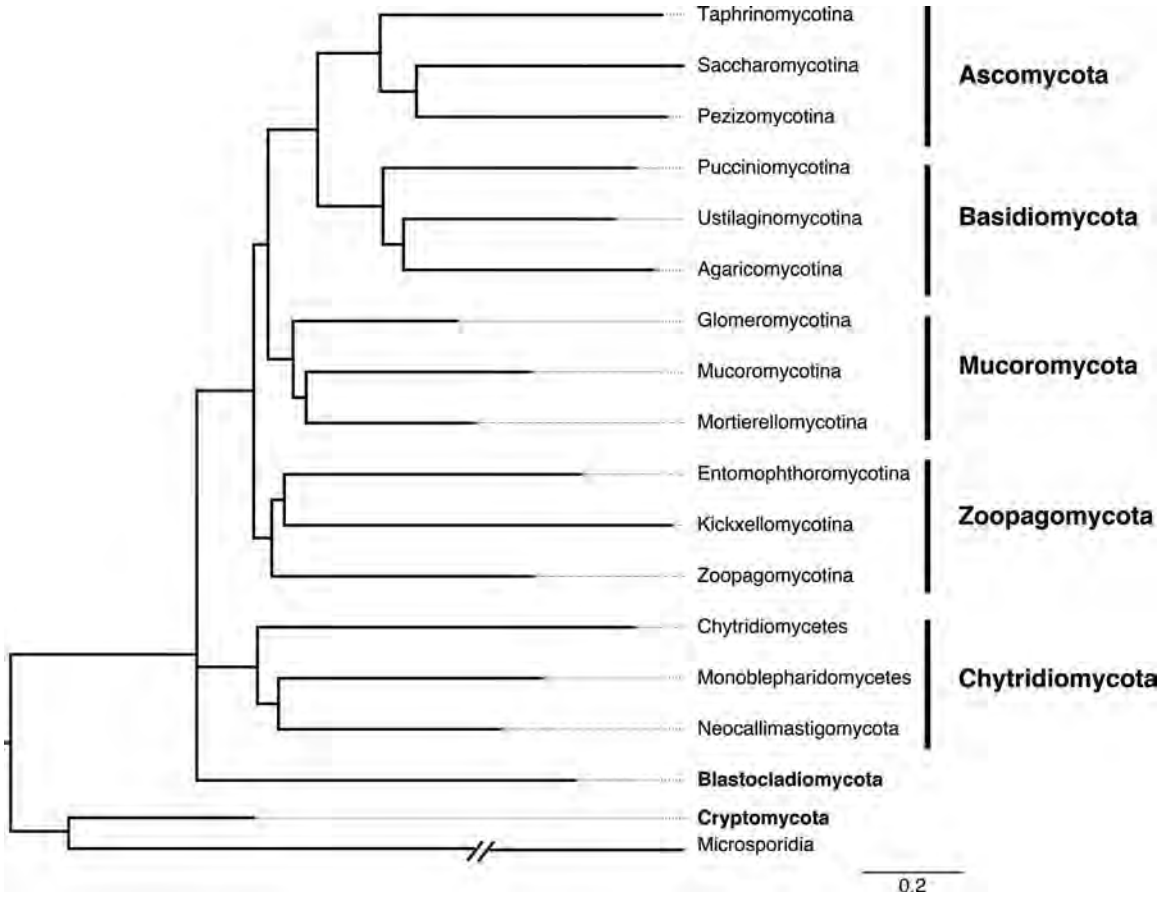


Figure 1 Phylogenetic relationships of the fungal phyla and subphyla. A phylogenetic tree from 434 conserved protein-coding genes resolves the relationships of most of the known lineages of fungi. This tree is a simplified version of that presented by Spatafora et al. (43). Phyla are presented in bold and subphyla in regular type. The Chytridiomycetes and Monoblepharidomycetes represent lineages for which a subphylum is not yet named.

first eukaryotes to benefit from these technological advancements to support the application of phylogenomic methods. Phylogenomics uses multiple orthologous genes, each sampled from a species genome to construct a composite phylogenetic tree from a concatenated dataset or individual gene trees (36, 39–43). A large collection of orthologous loci mined from genome or transcriptome sequences enables phylogenomic studies and tests for conflicting phylogenetic signals. These conflicts may be caused by genomic loci with different evolutionary histories due to, for example, incomplete lineage sorting, horizontal gene transfer, or different selective pressures (40, 44–48). Identification of gene tree and species tree conflicts can help to generate the best representation of the species tree and reveal current or historical selective pressures on genetic loci.

IMPACTS OF ECOLOGICAL NICHE ADAPTATION ON FUNGAL GENOME EVOLUTION

Genome sequencing has enabled comparisons of gene content and sequence changes to begin to predict the likely molecular basis for traits and adaptations. As heterotrophic organisms, fungi obtain carbon and nitrogen from external sources. A variety of cooperative and parasitic associations of fungi with other organisms are found throughout the fungal kingdom. Saprotrophic fungi liberate nutrients by degrading organic matter in habitats ranging from soils, dead insects and plants, compost piles, animal dung, and water-damaged homes. Ectomycorrhizal and arbuscular mycorrhizal fungi form mutualistic partnerships with plants and trade plant-produced carbon in the form of sugars in exchange for

inorganic nitrogen, phosphorus, iron, and other minerals. Fungi can live as commensal associates of plants and animals, for example, as plant endophytes or asymptomatic skin fungi, but also can cause devastating diseases in animals and plants via multiple invasive strategies. Some pathogenic fungi produce toxin molecules or effector proteins to kill host cells or disable host defenses, while others are opportunistic pathogens that only cause disease when the host becomes sick or immunocompromised.

Ectomycorrhizal fungi have mutualistic lifestyles with plants that involve trading resources. Plant pathogens secrete enzymes to break down cell walls. These enzymes can induce a plant defense response (49, 50). Fungi engaged in mutualistic fungal-plant symbioses typically have a reduction in genes encoding enzymes for plant cell wall degradation to avoid eliciting a plant defense response (51). Instead, effectors including small secreted proteins are typically expressed in the ectomycorrhizal fungal-plant interface to establish and promote the two-way partnership (51–53). Arbuscular mycorrhizal fungi also are important plant symbionts contributing to plant health. The molecular components of the interaction and roles of arbuscular mycorrhizal fungi-produced effectors are still emerging areas of research (54, 55).

Historical biotic and abiotic interactions have shaped the evolution of genes and the genome organization of fungi. Through the comparison of fungal genomes, the impact of natural selection and neutral processes has been assessed to indicate genes and genomic regions of importance in the evolution of species. For example, wood-degrading fungi are classified as brown or white rot fungi depending on their ability to degrade recalcitrant lignin polymers. The woody material in trees and bushes is made up of cellulose and lignin, the latter providing strength to plants. Lignin is highly recalcitrant to degradation, and when linked to hemicellulose and cellulose it can be inaccessible to ligninolytic enzymes. To access the lignin, white rot fungi secrete a combination of enzymes and organic acids to break down and later absorb carbohydrate degradation products of cellulose, hemicellulose, and lignin (56). Comparative analysis of the genomes of brown and white rotters found that metabolic and enzymatic gene families varied. The presence of specific families in the brown or white rot species was consistent with their classification and the chemical reactions these fungi can induce to break down woody material (42, 57–60). However, additional analyses have identified that some species of white rot fungi have unexpectedly missing genes that would typically predict that they were unable to degrade lignin (61). Exploration of the genomes of two white rot fungi indicated that despite similar lignin degradation capabilities, the species contained different enzyme classes, and this suggests that additional pathways of delignification exist and remain to be discovered (62).

Fungi can exist in extreme environments such as the anaerobic rumen stomachs of ruminants (63, 64), the dry Atacama Desert in Chile (65, 66), and hypersaline salterns with NaCl concentrations up to 30% (67) and as endolithic inhabitants of rocks in Antarctica (68, 69). Thermophilic fungi that grow in the desert can survive at temperatures above 60°C (70, 71). Studies of these thermophiles and their genome sequences have led to the development of new enzymes for biotechnology and cell biology studies due to their thermostability (72–74). Molecular adaptations that have allowed these fungi to colonize these extreme niches are still being uncovered, and genomic sequencing and comparisons among relatives of extremophiles should provide candidate genes that impact these abilities.

EVOLUTION OF GENOME SIZE

Genome sizes of fungi can vary by almost 3 orders of magnitude. A sampling of 325 phylogenetically diverse fungi has shown that genome sizes vary from 2 Mb in Microsporidia to 2 gigabases (Gb) in Pucciniales fungi, with a median of 35 Mb (https://github.com/1KFG/genome_stats/; Fig. 2). This vast range is the result of multiple genome reduction and expansion events. The total number of predicted protein-coding genes in these fungi varies by an order of magnitude, ranging from 1,800 genes in *Encephalitozoon* (Microsporidia) to 35,000 genes in *Sphaerobolus* (Agaricomyoctina), with a median gene count of 11,000. Some of the smallest and most compact genomes can be found in the obligate parasites Microsporidia, with sizes in the 2 to 6 Mb range (75–78). The free-living yeasts in the Ascomycota and the Cryptomycota parasite *Rozella* typically have genomes in the 7 to 12 Mb range (79–83), and the Basidiomycota *Cryptococcus* yeasts are around 20 Mb (83–85). Lineages containing yeast-forming species tend to have smaller genomes (such as *Schizosaccharomyces* and *Ashyba*), but this apparent reduction has occurred independently and multiple times in fungal history (36, 37, 86).

At the upper end of the range of currently sequenced species are the 150- to 175-Mb genomes of *Cenococcum geophilum* (Dothidiomycetes; Ascomycota), *Sphaerobolus stellatus* (Agaricomyoctina; Basidiomycota), *Tuber melanosporum* (Pezizales; Ascomycota),

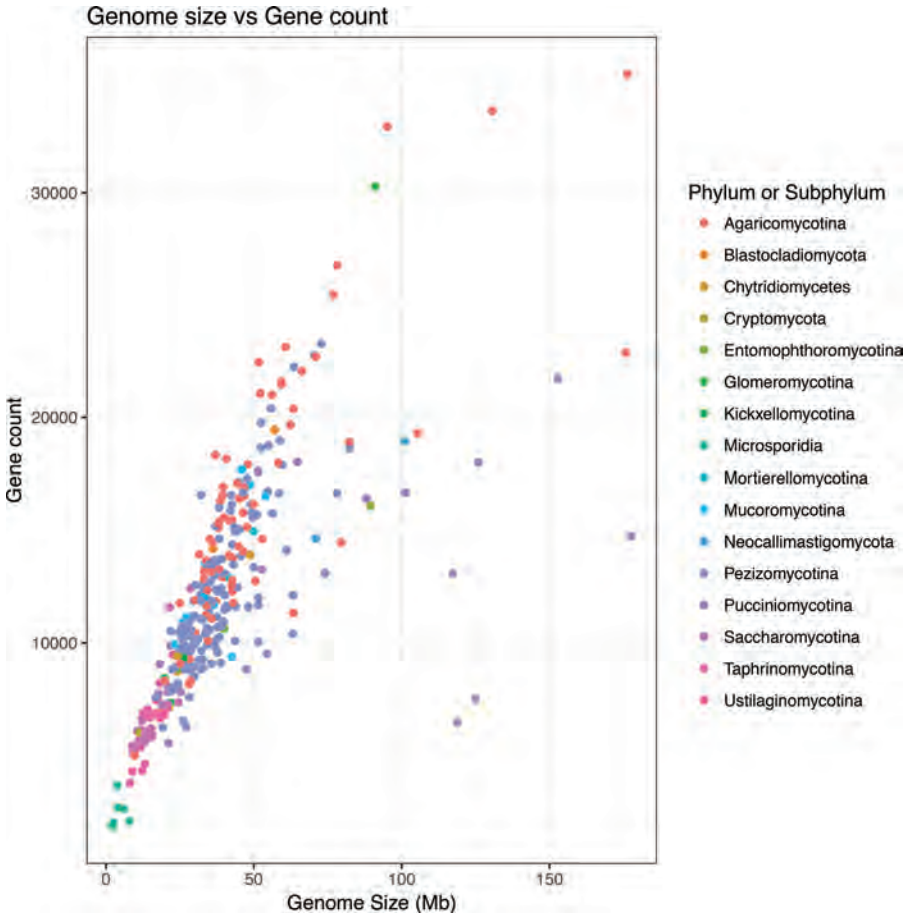


Figure 2 Scatter plot showing the relationship between genome size and gene count. Genome size varies among subphyla of fungi, with some of the smallest genomes in the Microsporidia and the largest currently sequenced genomes in the Agaricomycotina and Pezizomycotina. Primary data are gathered from genome information available at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) and Joint Genome Institute Mycocosm (https://jgi.doe.gov/fungi) and archived in the 1KFG genome_stats github project (https://github.com/1KFG/genome_stats).

and *Blumeria graminis* (Leotiomycetes; Ascomycota) (51, 87–89), which are primarily plant-associated fungi with both biotrophic and mutualistic lifestyles. Genome expansions in these species appear to have been driven mostly by transposon element content expansion. The total gene count is also higher in these genomes, which may belie an increase in gene duplication or insertion events that accompany the evolutionary processes that enabled increased transposon copy number.

Genomes of Entomophthoromycotina (Zoopagomycota) also are extremely large. The genome of *Entomophaga aulicae* is estimated to be as large as 8 Gb based on estimates using nuclear staining approaches (90), though sequencing has not yet been attempted. The genomes of the insect-killing zygomycete fungi *Entomophthora muscae* and *Zoophthora radicans* (Entomophthoromycotina; Zoopagomycota) appear to be in the ~700 Mb to 1.5 Gb range based on sequencing done through the Joint Genome Institute for the 1000 Genomes Project and others (M.B. Eisen and H. de Fine Licht, personal communication). The large genome size appears to be driven by increases in transposable elements, while gene count is not substantially expanded based on RNA-seq of these and related species (91, 92). Genome size estimation using flow cytometry has indicated that many of the rust fungi (Pucciniales; Pucciniomycotina) also have large genomes with estimated sizes of 300 to 900 Mb (93).

Genome reduction has also occurred in several fungal lineages, with the highly reduced genomes of the obligate parasitic microsporidian fungi being some of the most prominent examples, with tiny genomes (by

fungal standards) in the 2 to 6 Mb range (75–78). However, not all Microsporidia have tiny genomes; some are in the 16 to 20 Mb range, which seems to be due to transposon insertions in the few cases that have been examined (94). A smaller genome lends itself to fewer genes; as such, Microsporidia have a highly reduced gene set, dispensing with most small-molecule production and energy production pathways in favor of uptake transporters to obtain these resources from hosts (78, 95–97). In addition to fewer genes, the lengths of gene sequences themselves are reduced because the selective pressures that resulted in reduced genome size also contributed to reduced gene length (76, 95, 98). Coding space is at such a premium, with genes found nearly every 1,000 bp in some species (75), that some transcribed genes overlap (98, 99). Other obligate parasites in the fungal kingdom such as *Pneumocystis* demonstrate reduced genomes in the 8 Mb range, with approximately 3,700 genes (100–102), suggesting the presence of common selective pressures to reduce genome size as part of a tight host association in some parasitic fungal-host interactions.

Reduced genomes are a hallmark of yeast-forming lineages. Prominent examples include the Saccharomycotina yeast *Ashbya gossypii*, with a 9-Mb genome and 5,300 genes, and the Taphrinomycotina fungus *Schizosaccharomyces pombe*, also known as fission yeast, with a 12-Mb genome and 5,100 genes (80, 86, 103). The fern pathogen *Mixia osmundae* (Pucciniomycotina; Basidiomycota) has a 14-Mb genome (82) and 7,000 genes, and the human pathogenic *Cryptococcus* yeasts (Tremellomycetes; Basidiomycota) (83–85) have 19-Mb genomes with around 7,000 protein-coding genes. In each of these clades of yeast-forming fungi the genome sizes tend to be smaller than their filamentous sister clades. The filamentous Pezizomycotina fungi, sister to the Saccharomycetales yeasts, have genomes in the 35 to 45 Mb range, and the Agaricomycotina fungi sisters to the Pucciniomycotina are typically in the 45 to 60 Mb range. Analyses of gene gain and loss in yeast lineages have revealed that major gene losses occurred in the evolutionary history of these lineages, indicating genome reduction in the transition to single-celled growth (36, 37, 86).

Efforts to study how changes in gene content and evolution affect different lineages have identified classes of genes and genomic regions that change at different rates (104, 105). Within a genome, gene family copy number dynamics, transposable element transposition frequency, and individual gene evolution vary across a genome, which could be a consequence of different selective pressures but which also influenced random genetic drift. An important driver in the importance of random genetic drift versus selection in shaping a genome is the effective population size of a species. An organism's mode of dispersal, outcrossing frequency, and rapidity of cell division can all influence the effective population size, though the driving factors that determine effective population size are not well explored in fungi. Some of these regions, such as effectors and defense genes, can be important pathogenic or symbiosis-associated genes. The processes that establish and maintain different rates of change are likely not universal, but genome regions with extremely high rates of molecular evolution are particularly common in plants, fungi, and oomycete species (106–111) and may be important sources for novelty within species.

Many of the studies of genome size change are limited to lineages that diverged many millions of years ago. This divergence does not allow for the study of the mechanisms of genome size changes occurring at the population level. While aneuploidy and polyploidy manipulation and large-scale acquisition of DNA have been explored in *Saccharomyces* (112–115), studies of recent changes in genome size by massive gene duplications or transposon proliferation could reveal fitness consequences and the relative role of neutral versus directional selection in the success of lineages with genome size expansions (116, 117). Increased genome size may also result from changes in effective population sizes leading to relaxed selection and genome size increase (116). What might drive changes in population sizes? A newly acquired plant-associated lifestyle may impose constraints on reproductive modes (e.g., timing and availability of partners) or dispersal (e.g., how spores are produced). A better understanding of the pressures that drive genome size change could help us to understand how demography and transposable element proliferation influence fungal evolution, and in some cases how they have enabled the emergence of many disease-causing plant pathogens.

Gene and Gene Family Size Dynamics

Sampling entire genomes has allowed for increased resolution regarding the relationships between fungal lineages as well as the history of individual genes. Reconstruction of individual trees for each gene in the genome can provide a means to establish the age and coalescent history of a gene; e.g., a gene could originate with the Eukaryotes, in the Animal-Fungi ancestor, or be fungal-specific. Tools such as PhylomeDB and Ensembl Genomes Compara provide interfaces to explore these reconstructions to understand the history of a single gene (118, 119). Other events that generate

copy number expansions of a gene or gene family through duplication can be identified, and the timing of these duplication events can be established when comparing the copy number of the same gene family across a phylogeny of species with sequenced genomes. Likewise, a comparison of gene family contractions and gene losses can be studied to identify when potential function or diversity was lost. These changes in genome content provide important insights into adaptations that organisms may experience due to shifts in ecological niches or associations with a plant or animal host.

Gene family expansions

Comparisons of gene families between species have revealed several instances of gene copy number expansions possibly driven by evolutionary adaptations; such expansions have been instrumental in the evolution of pathogenicity traits. Copy number expansions of fungal genes containing the carbohydrate-binding LysM motif have been documented in both animal and plant pathogens (120–123). LysM protein motifs bind chitin or chitin-like carbohydrates and peptidoglycans (120, 124), and their role in fungi may be to bind the chitin in fungal cell walls to avoid triggering recognition by the plant or animal host defenses (125). The expanded copy number of these genes and the domain may be a signature of species that have biotrophic interactions with a host, and recent expansions could indicate a recent shift to this association from a saprotrophic lifestyle. The genomes of the human pathogenic fungi and basidiomycete yeasts *Cryptococcus neoformans* and *Cryptococcus gattii* show expansions of sugar and major facilitator superfamily genes (83, 84) hypothesized to play a role in increased uptake of these molecules from the environment (126) and could be important in synthesis and transport of the prominent capsule that is composed of the polysaccharides glucuronoxylomannan and glucuronoxylo-mannogalactan (127–129).

Expansion of the subtilase and metalloprotease gene families has also been noted as important in the transition to an animal pathogenic lifestyle in the filamentous ascomycetes Onygenales (33, 35, 130). The M36 metalloprotease family has expanded in *Coccidioides* fungi and their close relatives and is hypothesized to be linked to the switch from plant- to animal-associated ecologies and the switch from obtaining nutrients from plant-based carbohydrates to animal keratin and proteins (33, 34, 131). Research in animal-associated dermatophyte fungi has revealed similar, but typically independent, expansions of proteases in *Blastomyces* (132), *Trichophytoni*, and *Microsporum* (123). Interestingly, the human pathogen *Sporothrix*, not an Onygenales fungi but a member of a different clade (Ophiostomataceae; Sordariomycete), does not show signatures of recent protease family expansion, suggesting a different transition to mammalian association.

Comparison of the genes in the amphibian disease-causing chytrid fungus *Batrachochytrium dendrobatidis* to a nonpathogenic relative also identified expansions within protease families (133). The copy number of aspartyl and multiple metalloprotease gene families and the chitin-binding domain CBM18 are dramatically expanded in the pathogen as compared to nonpathogenic sister species (133). The importance of these expansions in pathogenesis is suggested by several investigations. One study found that a subtilisin-like serine protease was upregulated in response to amphibian host expression of thyroid hormone necessary for amphibian development (134). The CBM18 gene has the highest copy number of any fungus, ranging from 65 to 90 copies among sequenced strains, and has been under recent positive selection, indicating that it may be an important contributor to its pathogenicity (135, 136). Cloning and expression of the domain demonstrated that it also is capable of binding chitin (137). Comparison with the closest relative, *Batrachochytrium salamandrivorans*, also a successful and recently emerging pathogen, supports a hypothesis that the timing of these protease and CBM18 gene family expansions coincides with the emergence of the two *Batrachochytrium* lineages (136).

Plant pathogenic fungi have also undergone gene copy number expansion, effectively enabling host colonization and the ability to overcome plant defenses. One example is secreted effector proteins, which are expanded and diversifying in rust genomes (Pucciniomycotina; Basidiomycota) (138). Recent transposable element expansion, which is associated with gene duplications and accelerated rates of effector gene evolution, is noted in *Leptosphaeria maculans* (Pleosporales; Dothideomycetes) (106, 110). Other members of the *L. maculans* species complex have small genomes and lack this genome expansion. The powdery mildew *B. graminis* has a large genome with an expansion of atypical avirulence genes and signal peptide-containing genes (89). Metalloproteases and alpha amylases are expanded in the important plant disease-causing fungus *Zymoseptoria tritici* (formerly *Mycosphaerella graminicola*) (Capnodiales; Dothideomycetes). In contrast, contraction of CAZy family genes in *Z. tritici* suggests a specialization on the types of carbon sources utilized for nutrition (139).

Recent gene gains and losses have occurred during the evolution of insect-associated fungi, and these

changes appear to impact host specificity. The transition between generalist and specialist in *Metarhizium* species may have been driven by gene family contractions (140, 141). The "domesticated" fungus cultivated by leaf cutter ants, *Leucoagaricus gongylophorus*, has an expansion of CAZymes related to polysaccharide degradation. These enzymes are differentially expressed depending on the plant substrates "fed" to the fungus garden by leaf cutter farming ants (142–148). The specialized gongylidia that are swellings of the hyphal tip have evolved to provide a sugar nutrient source for the ants in exchange for the input of leaf and plant material into the fungal garden. The expansion and specialization of the fungal enzyme families necessary for rapid extraction of carbohydrates from plant material have likely been driven by this highly mutualistic symbiosis (146–148).

Expansions are not always linked to pathogenicity. The extensive light-responsive nature of the zygomycete *Phycomyces blakesleaanus* (Mucoromyoctina; Mucoromycota) is likely the result of the expansion of signaling pathways (149). These signaling protein expansions are also seen in the relatively closely related species *Rhizopus delemar* (previously identified as *Rhizopus oryzae*) (150), which may have enabled these coprophilic fungi to optimize the timing of fruiting and spore maturation in sync with the ephemeral ecology of a dung. Producing and orienting spore-forming structures at the right time of day can maximize the probability that offspring will be dispersed and establish the next generation. Genes encoding hydrophobin proteins, which are important for fruiting body development in mushrooms, are also expanded in copy number in Agaricomycotina species (151–154). The p450 monoxygenase family is also highly expanded in mushroom-forming fungi, especially in white rot fungi such as *Phanerochaete*, which likely allow it to degrade a rich collection of substrates including lignin (62, 151, 155). These expansions may have been important in niche adaptations and the evolution of specific traits such as lignin modifications and degradation, decomposition of complex hydrocarbons, and the ability to grow as a biotrophic plant pathogen (156–162).

Gene family contractions

Gene family contraction and gene loss have contributed to the evolution of fungal genomes. Some of these changes can be correlated to recent shifts in ecology, while other analyses have revealed ancient changes that appear to underlie a simplification in growth morphology. Researchers undertook a comparative analysis of 59 fungal genomes and examined changes in gene families corresponding to five independent lineages that grow primarily as single-cell yeast forms. The analysis used a newly developed method called COMPARE (comparative phylogenomic analysis of trait evolution) to infer that evolution of yeast growth forms occurred by convergent evolutionary processes leading to parallel, independent gene family losses (36). The predominant pattern of observed losses was in plant cell wall-degrading enzymes, fungal lysozymes, p450 families, and cyclophilins that serve as molecular chaperones.

Gene family contractions are evident in the plant pathogen *Colletotrichum* (Glomerellales; Sordariomycetes) and are associated with host range contractions (163), suggesting that host specificity may be a result of gene losses. Extensive gene losses were noted in the genome of *Escovopsis weberi*, an ascomycete pathogen of the *Leucoagaricus* ant-farmed basidiomycete fungi. These extensive gene losses in this species could result from specialization to mycoparasitism (164). Gene family contractions are not seen in the related mycoparasitic *Trichoderma* sp., suggesting a different route of host specialization in *Escovopsis*. Losses of gene families such as of dehydrogenases have been documented in the insect pathogens *Metarhizium* and interpreted to reflect adaptation to host specificity (141).

A lack of duplicate genes is not always a direct result of gene loss. In species in which repeat-induced point mutation (RIP) occurs, gene duplications cannot persist because RIP, a genome defense mechanism, targets duplicated sequences so that they accumulate point mutations during the meiotic cycle (165, 166). RIP is hypothesized to be a potent defense against transposable element proliferation because both the source and transposed copy of an element will become mutated. The genome of the ascomycete *Neurospora crassa* has been shaped by RIP. *N. crassa* does not have a reduced genome (40 Mb, ~10,000 genes), lacks nearly any active transposable elements, and also does not have any large gene families (167, 168).

GENOME STABILITY AND PAN-GENOMES

Gene content and genome copy number can vary, sometimes dramatically, in a population and across a species. Sequenced genomes represent a snapshot of the genomic information of a species or strain. The inventory of genes revealed by sequencing can be useful when comparing between isolates or sampling a population of individuals at different times. Changes in genomic content can occur among individuals in a population or even within a strain over time. Considering the complete set of genes across all strains or individuals of a

species is deemed the pan-genome (169), which can be a useful way to think about not just gene presence/absence compared to a "reference" but also about the complete set of genes that exists in a species or a collection of individuals (170). Genetic variation includes single nucleotide substitutions, insertion/deletions, or larger genetic content changes such as transposable elements and transposition events. The presence (1), absence (0), or amplification of copy numbers (>1 copy) in a gene can also be evaluated among individuals in a population. Together, these approaches can rank and identify quickly or slowly evolving genes and evaluate the evolutionary lability of genes and pathways which could underlie changes in function among populations and species.

Investigations have revealed that gene content and genome copy number can vary, sometimes dramatically, in a population and across a species. In *Saccharomyces cerevisiae*, completed genomes of 100 individual strains have been used to generate a pan-genome of fungal genes, some of which are present in some but not all strains (171). Changes are more common, but not exclusive to subtelomeric regions of chromosomes. Variation in the tempo and mode of chromosomal rearrangements and gene shuffling can be seen in wild and domesticated species when comparing *S. cerevisiae* and *S. paradoxus* (172) and wild or domesticated *Aspergillus* strains (173, 174). The human pathogen *C. gattii* has genomic segments that vary in copy number, segregate in the population, and could be a contributing mechanism to virulence differences among strains (85, 175).

The importance of genome variation at the population level is also appreciated in plant pathogenic fungi where variation in virulence and prevalence from year to year can sometimes be linked to a single gene or to changes in chromosome content. Genes encoded on dispensable chromosomes are sources of rapid changes in gene content because these chromosomes can be transferred between individual strains and often lost without disruption of primary metabolic or cellular functions (176–178). Also known as accessory chromosomes, these may accumulate transposable elements and evolve more quickly than other chromosomes because they typically do not encode essential genes (108, 179). These chromosomes further support a mechanism of gaining or losing sets of genes that may be important in adaptation to new host plants (178). The relative importance and molecular mechanism impacted by these genetic elements in modulating adaptability of species remains to be explored but can be important contributors to genome evolution.

CONCLUSIONS

Complete sequencing of fungal genomes has enabled comparisons of the dynamic genome size and gene content across a range of time in fungal evolution. Genome differences that are also identified through comparative genomics can help to form hypotheses about molecular mechanisms for adaptation to new ecological niches or fungal-host specialization. These identified changes establish guides for further genetic and molecular biology experimentation. Genome content comparisons also highlight the relative lability of fungal processes: core metabolism changes little, but copy numbers of transcription factors, secondary metabolites, and transporter families ebb and wane across the kingdom. Sequencing and analysis tools have permitted the detailed cataloging of where and how much change occurs across genomes, providing rationale for molecular experiments to study the functions of the genes implicated.

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Sources of Fungal Genetic Variation and Associating It with Phenotypic Diversity

30

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SOURCES OF FUNGAL GENETIC VARIATION

Genetic variation is the stuff of evolution: if there is no variation, there can be no evolution. This review of fungal genetic variation begins with a survey of its sources and then discusses means of associating natural genetic variation with phenotypic variation, including phenotypes that are important to fungal adaptation or those that interest cell and developmental biologists engaged in basic or translational research. The tale of the study of fungal genetic variation is also a tale of advances in genomic science, and it is appropriate to note that the first Eukaryote to have its genome sequenced was a fungus, the model yeast *Saccharomyces cerevisiae* (1). Shortly thereafter, yeast was joined by filamentous Ascomycota, e.g., *Neurospora* (2), and Basidiomycota, e.g., *Phanerochaetae* (3). Not long after, three *Aspergillus* species were sequenced—*Aspergillus oryzae* (4), *Aspergillus fumigatus* (5), and *Aspergillus nidulans*—and the comparisons of their genomes (6), along with those of yeasts closely related to *S. cerevisiae* (7), represent landmarks in the field of comparative fungal genomics. A survey taken in April 2017 of fungal genomicists associated with the Joint Genome Institute and FungiDB estimated the number of fungal species with sequenced and assembled genomes at 2,000 and estimated that another 1,000 to 1,500 genomes represent multiple individuals from species that are studied by population genomics. No other group of eukaryotes enjoys as deep a genomic database as is seen for the fungi.

Gene Gain and Loss

The sources of genetic variation covered in this review are listed in Table 1. One of the first types of genetic variation discoverable by comparing genomes was gene gain and loss by duplication or excision. Again, the model fungus *S. cerevisiae* was involved because it showed abundant gene duplication. The debate that ensued over the source of gene duplication, whether by whole-genome duplication (8) or by the accrual of smaller duplications (9), was settled in favor of whole-genome duplication when genomes of outgroup yeasts were analyzed (10, 11). This episode made two seminal contributions that will be revisited in this article: that interspecific hybridization is an important source of genetic variation in the Fungi (12, 13) and that duplications as large as chromosomes are routinely gained and lost throughout the Fungi (14, 15).

Most duplications are rapidly lost, and where duplicated regions are retained, natural selection is likely responsible, as will be seen below. As more representatives of Ascomycota were sequenced, broader comparisons became possible (16). These comparisons revealed abundant examples of gene gain and loss and made two additional discoveries. First, duplications are more common in proteins that respond to stress than in those involved in core metabolism and, second, where duplications are retained, they result in the evolution of differential expression of the paralogs more often than the evolution of new functions. Where the duplicated protein formerly participated in more than one pathway,

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Table 1 Genetic variation and its use in associating genotype and phenotype

Sources of genetic variation in fungi
Single nucleotide mutations and recombination (76–82, 98)[a]
Gene gain and loss (7–17)
Gene family expansion and contraction (20–26)
Horizontal gene transfer (33–38, 45–53)
Selfish DNA (39–43)
Aneuploidy (54–60, 148)
Conditionally dispensable chromosomes (59)
Loss of heterozygosity (61–64)
Genome rearrangements (18, 19, 65–75)
Epigenetic modifications (83, 84)
Endofungal bacteria (85–90)
Fungal viruses (91–94)
Mitochondria (95–97)
Hybridization and introgression (111–141)
Using natural variation to associate genotype and phenotype
QTL, GWAS, and reverse ecology (98–110)
Hypothesis testing in wild fungi (142–147)

[a]References germane to topic.

duplication allowed the copies to evolve to focus on just one pathway or the other (16). This disentangling of pathways may explain why more duplicated genes are retained, and in a recognizable form, than would be predicted by evolutionary theory that assumed retention would require neofunctionalization (17). Although gene gain clearly gets more attention than gene loss, when it comes to plant-pathogenic fungi, gene loss can be an important contributor to infection of novel hosts through the loss of genes whose products engage host defenses (18, 19).

Gene Family Expansion and Contraction

As regions of the fungal tree of life became more densely populated with whole genomes, comparative phylogenomics uncovered gene family expansions and contractions that could be correlated with adaptation (Fig. 1). Using an approach developed for all eukaryotes (20), plant pathologists compared the rice blast fungus, *Magnaporthe*, with *Neurospora* and *Aspergillus* and found expansion in nearly 10 gene families, including those associated with pathogenesis, e.g., cytochrome p450 genes and G-protein-coupled receptors (21). Subsequent addition of genomes expanded the search to more pathogens and the discovery of more expanded gene families including those whose products populate the secretome (22). The approach was refined (23) and expanded to animal-pathogenic fungi, where the shift from eating plant cell walls to eating animal protein was documented by expansions in genes coding for proteinases and contractions in those coding for cellulases and other enzymes that deconstruct plant cell walls (Fig. 1) (24). Gene family expansion and contraction have even been applied to major shifts in the geologic record, such as the end of the coal age (25). Here, while studying genes coding for enzymes that saprotrophic fungi used to decay lignin, mycologists found an expansion that they could correlate with the end of the Carboniferous age, thereby supporting speculation that these fungi helped spell the end of widespread coal formation. Of course, expansions and contractions are relative events, and a caveat in the search for gene family expansion and contraction was recently sounded, where what was thought to be an expansion of genes coding for proteins with LysM domains in a group of dermatophytic fungi proved rather to be a contraction in a related fungal clade that harbors the systemic pathogens *Coccidioides immitis* and *Coccidioides posadasii* and their nonpathogenic relatives (26).

Interkingdom Horizontal Gene Transfer

The same genomes that permitted the discovery of gene duplication within a genome also permitted the discovery of genes gained from outside the species by the process of horizontal gene transfer (HGT). An early and prescient review discussed the possibilities in fungi (27) at a time when HGT was being studied in whole genomes of prokaryotes (28), and there have been a number of subsequent reviews (e.g., 29). To demonstrate HGT, it is necessary to show that the phylogeny of the transferred genes is truly in conflict with the organismal phylogeny as inferred from other genes. Before genomes, it was not easy to demonstrate HGT, but whole genomes provide more than enough data to achieve a statistically significant result. Plant-fungal HGT has been shown to work in both directions and, although infrequent and apparently ancient, exchanges of transport proteins and siderophores are thought to have aided the invasion of the terrestrial environment by both fungi and plants (30). Far more common are HGT events between the kingdom Fungi and Oomycota; here it can be fairly said that HGT from Fungi, mostly Ascomycota, enabled Oomycota to become plant parasites (31). Among the genes transferred from Ascomycota to Oomycota are those that code for proteins responsible for deconstruction of plant cell walls, uptake of nutrients, resistance to plant defenses, and the aggressiveness of the parasitism. Traffic from Oomycota to Fungi is far less frequent, with the best example being a monooxygenase-like protein involved in antibiotic synthesis (31).

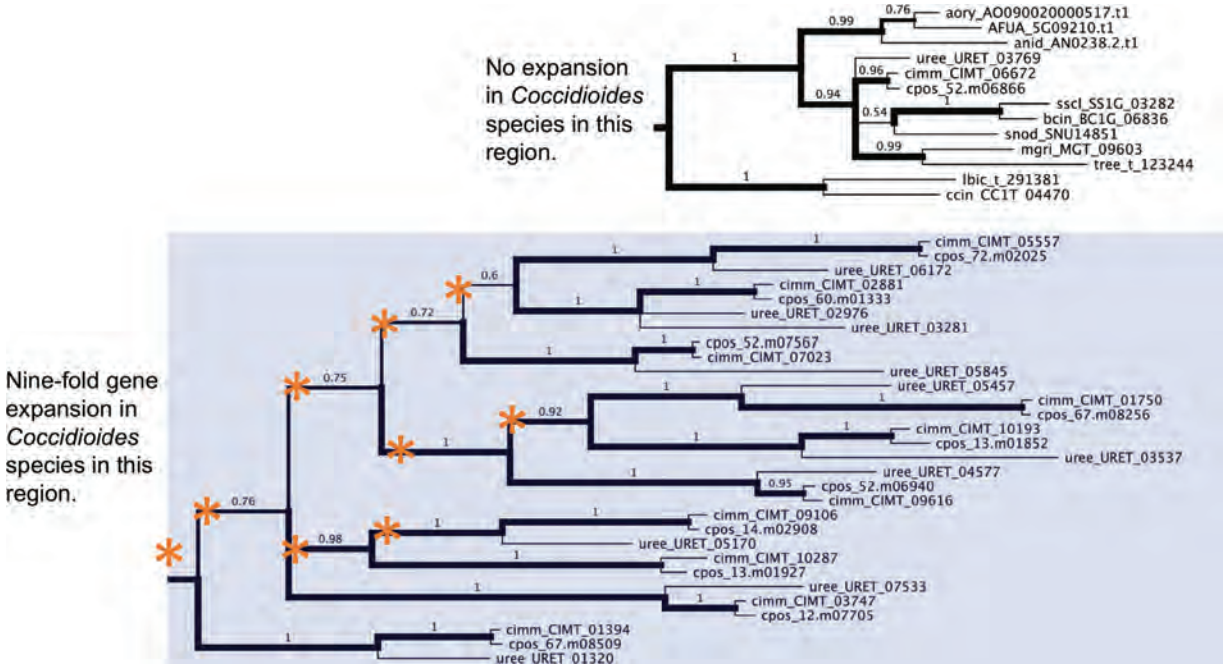


Figure 1 Gene family Bayesian phylogeny for proteinase genes with S8 domains showing (top) some phylogenetic lineages with no expansion and (below) others with a large expansion due to nine gene duplications (asterisks). Key to taxon abbreviations preceding gene identifiers: *Aspergillus oryzae* (aory), *Aspergillus fumigatus* (afum), *Aspergillus nidulans* (anid), *Uncinocarpus reesii* (uree), *Coccidioides immitis* (cimm), *C. posadasii* (cpos), *Sclerotinia sclerotiorum* (sscl), *Botrytis cinerea* (bcin), *Stagonospora nodorom* (snod), *Magnaporthe grisea* (mgri), *Trichoderma reesii* (tree), *Laccaria bicolor* (lbic), and *Coprinopsis cinerea* (ccin). Adapted from reference 24.

Fungi living in the guts of animals also experience HGT, and one of the most remarkable examples of interdomain HGT concerns the Chytridiomycota that live in the stomachs of ruminants. These fungal symbionts clearly received glycosyl hydrolase genes from bacteria, a transfer that helped them adapt to feeding on plant cell walls in the stomachs of ruminants (28). Gut fungi in the Zoopagomycota also have experienced HGT in the form of a ubiquitin gene received from their mosquito hosts (32). Could these genes, known to mark proteins for degradation or to alter their function or location, be involved in manipulating the host? As related above, interkingdom and interdomain HGT is well documented, but the mechanisms of gene transfer among distantly related organisms are not understood.

Transposable Elements

Not all HGT involves genes that confer an advantage to the recipient, at least initially. Some involves selfish nucleic acids, and the most prevalent types are transposons and retrotransposons, which are capable of moving and copying themselves in one genome or among genomes (33–35). Transposable elements (TEs) provide genetic variation by disruption of existing genes and gene order, by stimulating rearrangements and duplications, as well as by simply adding genetic material that can then evolve to new functions. Many fungi have an active defense against TEs, e.g., repeat induced point mutations (RIP) (36). RIP targets regions of repeated DNA sequence and in both copies mutates C-G pairs to A-T, sometimes spilling over into an adjacent, unduplicated sequence. Thus, it can reduce genetic variation by restricting gene duplication and also enhance it by generating mutations (37). However, the strength of RIP varies widely among fungi, as does the presence of TEs, and many symbionts, whether mutualists, i.e., ectomycorrhizal fungi, or pathogens, have large genomes due to an abundance of these mobile elements (38). A recent study of a Basidiomycota plant parasite, *Microbotryum*, showed that, despite RIP, TEs show bursts of activity that periodically increase the size of fungal genomes (39). Another recent study, focused on the Ascomycota plant pathogen *Fusarium*, investigated the role of TEs in a type of aneuploidy that will be discussed below (40). A different type of TE is a homing endonuclease, which enters recipient genomes at

extremely conserved sites, e.g., ribosomal RNA genes, and which spreads faster than Mendel imagined by being transmitted to all progeny. Homing endonucleases have a "life cycle" of transmission to a new species followed by the spread among the individuals of the new species until all available invasion sites are filled. At that point, there can be no additional transmission and no selection for active endonuclease, so the elements degenerate and disappear from the species, making it ripe for new introduction (41, 42). Work on these elements in yeast led to a proposal that homing endonucleases could be used to extirpate insect vectors of disease, such as the mosquitoes that vector malaria (43), an idea that has gained popularity with the application of the CRISPR-associated protein-9 nuclease system to genetic manipulation (44).

HGT Among Fungi

Fungus-to-fungus HGT of coding genes is also a source of genetic variability involved in adaptation, and none is more spectacular than the transfer among Ascomycota of a toxin gene, *ToxA*, that instantly confers on the recipient the ability to attack wheat cultivars that possess the toxin-sensitive allele of the plant gene *Tsn1* (45). In this case, the direction of HGT could be determined by assessing, in the two fungi, the amount of genetic variation in the transferred toxin gene, *ToxA*. Using the logic that the more variable species was the donor and the species with little to no variation was the recipient, the flow could be shown to be from *Parastagonospora nodorum* to *Pyrenophora tritici-repentis* (45). Remarkably, the plant gene targeted by the toxin is a member of a wall-associated class of kinase receptors that help plants resist rusts and biotrophic fungal pathogens by killing affected cells and starving the fungus. When the fungus is a necrotroph, as is *P. tritici-repentis*, the programmed plant cell death initiated by the toxin instead opens the plant to attack by the necrotroph—a heavy cost to maintaining resistance genes (46–48).

Among cases of fungal HGT, equally amazing as the ToxA story is the horizontal transfer of the multigene complexes that produce secondary products, or extrolites, e.g., polyketides such as aflatoxin or nonribosomal peptides such as penicillin. Although most polyketides may be transmitted vertically (49), it is clear that these complexes experience HGT, and it is equally clear that the dense clusters of genes in the complexes and their position near telomeres facilitate their transfer (50). It even has been hypothesized that the genes are aggregated to foster their later spread by HGT (51). Although it is clear that production of a specific extrolite is less likely to be transferred if the genes responsible for its synthesis are distributed throughout a genome, it is difficult to understand how selection could act on a potential future event. The more prosaic explanation for the clusters, i.e., that natural selection favors gene clusters because their regulation is better coordinated, likely provides the selective advantage needed to keep clusters intact, as in the case of the massive clusters found in species of the Ascomycota, *Penicillium*, involved in cheese making (52, 53).

Aneuploidy

The advent of massively parallel methods of DNA sequencing, and the high throughput sequencing they support, gave mycologists the ability to obtain genomes from populations of individuals. The strains of fungi that are resequenced to enable population genomics can come from members of a natural, interbreeding population, from strains collected from a clonal outbreak of a pathogen spread by asexually produced spores, or from a series of strains that have evolved inside a single patient. It is the last of these collections, made from a single patient infected with the pathogenic yeast *Candida albicans*, that let genomics demonstrate the importance of aneuploidy, the possession of more or fewer chromosomes than normal, as a source of fungal genetic diversity (Fig. 2) (54). In this study, *C. albicans* strains that had been isolated from candidiasis patients undergoing treatment with azole antifungal drugs had an extra, aneuploid chromosome made of two copies of the left arm of chromosome 5. In this diploid yeast, this aneuploidy doubled the copies of efflux pumps that can export the drug, a transcription factor positively regulating the pumps, and the target of the azole drug, an enzyme in the ergosterol synthesis pathway. Studies of another human pathogenic fungus, the Basidiomycota *Cryptococcus neoformans*, showed that a different type of clonal reproduction, unisexual mating, whereby meiotic basidiospores arise from the fusion of two mitotically produced nuclei, can also generate aneuploid progeny (55). In this latter research, the authors documented shortening of chromosomes among strains, as well as chromosome duplications, and found that unisexual progeny displayed significant phenotypic variation for traits involved in virulence. These traits included temperature sensitivity (but without loss of virulence in mice), increased resistance to antifungal azole drugs, and increased production of the virulence factor melanin. The authors also showed that unisexual mating was not essential for the generation of aneuploidy because the phenomenon was also seen in biparental, sexual progeny of two different *Cryptococcus* species (55).

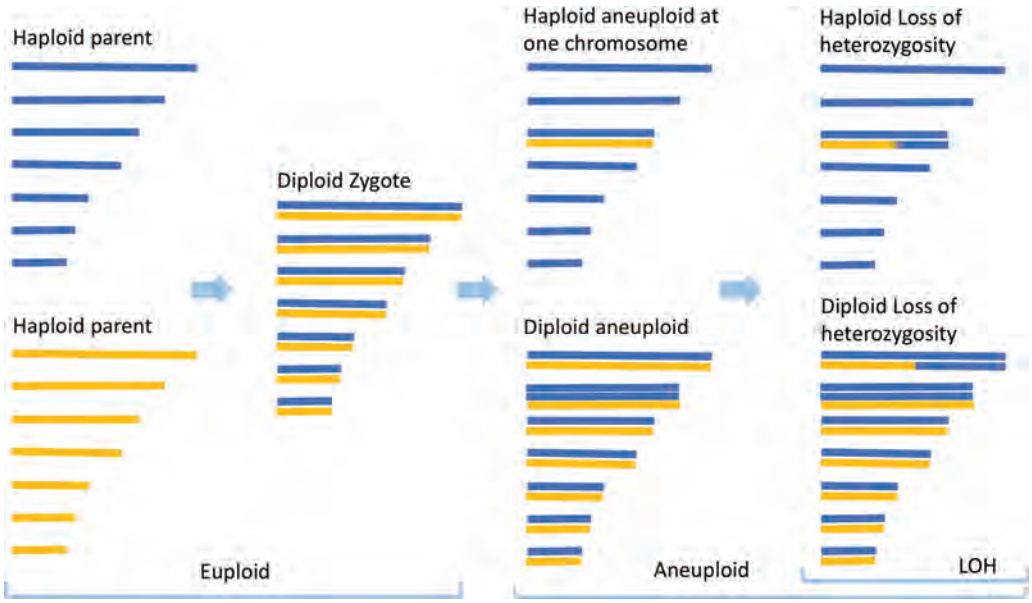


Figure 2 Diagrams showing aneuploidy and loss of heterozygosity (LOH) in haploid and diploid genomes. Each individual has seven distinct chromosomes colored to show heterozygosity.

Aneuploidy has also been shown to be involved in virulence in another animal pathogen, the Chytridiomycota that is responsible for amphibian decline, *Batrachochytrium dendrobatidis*. Amphibian decline has been correlated with the global pathogenic lineage (GPL) of strains obtained from infected animals. Aneuploidy is seen in both the GPL and, to a lesser extent, among individuals in the population from which the GPL emerged (56, 57). Data presented in both of these referenced studies show that passage of the fungus in the laboratory can result in loss and gain of aneuploid chromosomes and that genes known to code for virulence factors are hot spots for genomic change. More recently, comparison of the genomes of isolates from the same lineage, one original and the other having endured 6 years of laboratory transfers, also showed a correlation between reduced virulence and aneuploid loss of chromosomes (58).

Conditionally Dispensable Chromosomes

A different type of aneuploidy is seen in symbiotic Ascomycota, whether parasitic or mutualistic, that may have more chromosomes than do their nonsymbiont relatives. Unlike canonical aneuploidy, here the extra, conditionally dispensable chromosomes (CDCs) are different from, and in addition to, the chromosomes in the core genome (Fig. 3). Their presence varies among individuals in the species, and CDCs contain a disproportionate number of TEs. They may contain virulence factors not seen in the core genome, and they can be transmitted among species by hybridization, thereby conveying the ability to become parasites (59). A recent study with a well-assembled genome of *Fusarium poae* reported that the dispensable chromosomes lack an effective RIP, which explains their being riddled with TEs (40). This study also showed that CDCs act as a reservoir for duplicated genes and that the genes can then be transferred back to the core genome. Most of the fungi possessing dispensable chromosomes are plant symbionts, and one wonders if access to the abundant resources of a photosynthetic organism allows them to support an enlarged genome. Could this source of variation be a benefit of association with a domesticated plant, albeit an evolutionarily Faustian bargain given the danger of narrow host specialization? Alternatively, do fungi that have their pathogenicity factors in volatile regions of the genome respond more quickly to changes in host plant defenses and thereby avoid narrow host specialization?

Another type of unusual ploidy is demonstrated by hybridization among haploid, Ascomycota grass parasites in the genus *Epichloë*. These events, apparently not involving sexual processes, have produced diploid and triploid, asexual, mutualistic endophytes that are dispersed as hyphae in the seeds of their hosts. This unusual combination of aneuploidy and HGT, albeit among close relatives, has produced fungi that have a life history that is extremely different from their parental

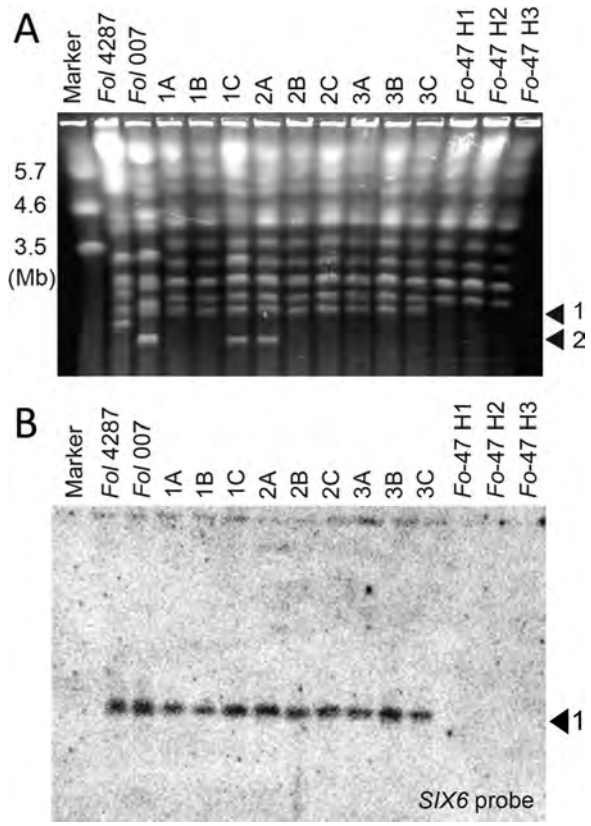


Figure 3 Contour-clamped homogeneous electric field gel karyotype of *Fusarium oxysporum* chromosomes showing conditionally dispensable chromosomes (CDCs) and their transmission between strains. (**A**) Donor strain *Fol*007 (left) harbors CDCs 1 and 2 (arrows), and recipient strain *Fo*-47 (right) lacks them. Strains 1A-3C (middle lanes) are derived from simple coincubation of *Fol*007 and *Fo*-47. These strains have the *Fo*-47 karyotype and have gained CDCs 1 or 2 (arrows), or both, from *Fol*007. (**B**) Southern hybridization of the contour-clamped homogeneous electric field gel to a probe with DNA from CDC 1 (*SIX6*), confirming the presence of CDC 1 in donor strain *Fol*007 and progeny strains 1A-3C, which possess the karyotype of the recipient strain *Fo*-47. Adapted from reference 148.

species, which are sexual, *Epichloë* species that transmit themselves horizontally via their ascospores and then aggressively parasitize and sterilize their grass hosts (60).

Loss of Heterozygosity

Where organisms are more than haploid, i.e., diploid, aneuploid, or polyploid, they are almost certainly heterozygous at many genes, itself an obvious source of genetic variation. These heterozygous organisms can lose heterozygosity through gene conversion or crossing over or other events that leave portions of homologous chromosomes genetically identical (61) (Fig. 2). At first it might seem that loss of heterozygosity would necessarily reduce phenotypic variation, but phenotypic diversity can be increased if dominant alleles are lost and release the activity of the recessive allele. Such a case could also occur through aneuploidy, where loss of one copy of a chromosome would expose recessive alleles on the remaining, now haploid, copy. An insightful example of this type of loss of heterozygosity was reported in *C. albicans* strains that are resistant to azole antifungal drugs due to the activity of multidrug efflux pumps. Here, it was mutations in the gene coding for a transcription factor on chromosome 3 (which regulates the aforementioned pumps on chromosome 5) that were responsible for the constitutive expression of the pump genes. *C. albicans* being diploid, and the mutant transcription factor genes being recessive, the phenotype was seen only when the dominant wild-type allele was lost. Selection by the azole drug favored strains of *C. albicans* that both had the mutation in one of the copies of the transcription factor and had lost the wild-type allele through aneuploidy (62).

One of the tenets of population biology, Haldane's sieve, states that adaptive alleles are more likely to be dominant because newly mutated alleles are rare and cannot experience positive selection unless they are dominant and support a heterozygous phenotype. However, adaptation due to recessive alleles being released from dominance by loss of heterozygosity was recently demonstrated in an experimental study employing *S. cerevisiae* strains under selection for resistance to the antifungal drug nystatin (63). Diploid strains were created that were either homozygous for a recessive allele that conferred resistance to nystatin or heterozygous with a wild-type, susceptible allele. When grown without the drug, fitness was not strictly correlated with hetero- or homozygosity. When, however, the strains experienced nystatin stress, the strains homozygous for the resistant allele outperformed heterozygotes unless loss of heterozygosity removed the dominant, susceptible allele from heterozygotes, showing how recessive alleles can be adaptive. Loss of heterozygosity has also played an important role in the evolution of the GPL of *B. dendrobatidis* by generating variation among individuals of the GPL through recombination associated with mitosis, alone or in combination with meiosis (64). Here, is it possible that the loss of heterozygosity has released recessive alleles that promote virulence in this amphibian pathogen?

Genome Rearrangement

Rearrangement of the genome is also a source of fungal genetic variation, and one that provides a mechanism for gene family expansion and contraction through

gene duplication, deletion, or disruption (65). In the Ascomycota class Dothideomycetes, comparison of rearrangements among the genomes of 18 representative taxa showed that most rearrangements were within chromosomes (66), an example of mesosynteny (67). The most intensive study of fungal genome rearrangements has focused on those associated with an absence of recombination, specifically in mating loci and mating chromosomes. In these regions, recombination in the mating locus would produce progeny unable to mate, a phenotype readily eliminated by natural selection. Studies of barriers to recombination associated with rearrangements have been enabled by well-assembled genomes in the Ascomycota *Neurospora tetrasperma* (68, 69) or in the Basidiomycota *Cryptococcus* (70, 71) and *Microbotryum* (72–74). An interesting but unanswered question is whether the rearrangements seen in these mating regions suppress recombination or whether recombination is suppressed by some other means, thereby allowing rearrangements to accumulate. Expect much more research on the impact of genetic rearrangements in the near future (75) as it becomes economically feasible to practice population genomics with the superbly assembled genomes provided by the new generation of long-read, single-molecule DNA sequencers.

Mutation and Recombination

Until this point, we have avoided mention of the obvious contribution of sexual recombination to fungal genetic variation, although mutation, mating, and recombination, which provide the ability to generate progeny with genotypes different from parents and siblings, are at the heart of genetic variation. So important is this contribution that large natural populations, as must be the norm for many fungi, are thought to have all possible mutations in their genomes as a result of a combination of recent, ongoing mutation and standing variation (76). A recent study of polymorphism in North American and Russian populations of the Basidiomycota *Schizophylum commune* showed that this fungus is the most genetically diverse Eukaryote yet discovered, due to a rapid mutation rate and likely a large population size (77). As diverse as *S. commune* is, it is not alone and is in the close company of roundworms and choanoflagellates (78). Despite all of the costs associated with mating and recombination compared to clonal reproduction, i.e., from finding a mate, to loss of well-adapted genotypes, to having to share half of the genomes of progeny with a partner, sex and recombination are omnipresent in fungi (79) because they accelerate adaptation to changing environments, as shown by careful experimentation employing *S. cerevisiae* (80). Variation, however, cannot be limitless because some genetic combinations must be deleterious and selected against under conditions encountered in nature. Evidence for these natural limits is seen when phenotypes of parents and their progeny from laboratory crosses are compared; the variation in the progeny typically exceeds that of the parents or the natural population from which they were sampled. This transgressive variation exhibited by the progeny is often seen in the progeny of artificial crosses for complex traits, for example, the fungal phenotypes of aggressiveness in a plant pathogenic fungus (81) or growth rate in a wood decay fungus (82). Sexual reproduction deserves more mention, and it will be taken up again, below, in the section on associating genetic and phenotypic variation.

Epigenetics

Epigenetics is another important source of variation in fungi, not only among individuals but also among hyphal segments in a single individual (83), and its true importance is likely not yet appreciated because no population study of epigenetic variation has been published. As with studies of rearrangements, studies of epigenetic variation are to be expected in the near future because the aforementioned new generation of long-read, single-molecule DNA sequencers also have the ability to assess methylation of nucleotides (84).

Endosymbiotic Bacteria

Endofungal, bacterial symbionts, although not part of the fungal genome *per se*, are also a source of genetic variation for fungi, and one whose importance is emerging. These endosymbionts had been discovered in arbuscular mycorrhizal fungi by microscopy (85), but the extent of their presence was not appreciated until genomic studies of fungi revealed bacterial DNA among the expected fungal sequences (86). Recent studies show a fitness advantage to fungi possessing the endobacteria (87) and a lack of genome degeneration in the endobacteria themselves (88, 89). Nor are endosymbiotic bacteria restricted to arbuscular mycorrhizal fungi; one well-studied endobacterium is in the Mucoromycota *Rhizopus microsporus*, where it produces a toxin essential for the ability of this fungus to parasitize rice (90).

Fungal Viruses

Less explored is the role of fungal viruses as sources of genetic variation in fungi. The best-studied case is a virus that reduces virulence and reproductive capacity in the tree pathogen *Cryphonectria parasitica* (91). An equally compelling example is a virus that, along with

its host Ascomycota, *Curvularia protuberata*, confers improved thermotolerance in this endophytic fungus and its host plant, *Dichanthelium lanuginosum*, allowing them to live in geothermal areas of Yellowstone National Park (92). Although it is clear that fungi have strong defenses against virus (93), a recent search of RNA and virus particles isolated from five well-known plant-pathogenic fungi using a metatranscriptomic approach found 66 previously undescribed mycoviruses (94). Surely, many additional virus sequences are lurking unrecognized in the unsequenced pool of fungal genetic material. The nucleic acid diversity found in viruses and endofungal bacteria, plus that found in another cytoplasmic feature, the mitochondrion, can contribute to phenotype directly and by interacting with nuclear diversity. The role of cytoplasmic elements in heritability of a fitness phenotype, growth rate, was recently explored in yeast (95) and might also prove valuable in studies of fungi where mating can create progeny with identical nuclei but different mitochondria, for example, Basidiomycota (96) or dikaryotic Ascomycota (97).

USING NATURAL GENETIC AND PHENOTYPIC VARIATION TO ASSOCIATE GENES WITH PHENOTYPES

Having discussed different sources of genetic variation for fungi, it is time to ask, Is there any practical value to this knowledge—that is, value beyond satisfying the insatiable curiosity of mycologists and, of course, providing the genetic variation that enables fungi to adapt to the ever-changing environment? A simple answer to this question is that the same genetic variation that facilitates the adaptation of fungi can be used to solve a problem faced by many modern biologists, i.e., finding the genetic features responsible for a phenotype of interest or, more generally, associating phenotypic variation with genetic variation.

The Standard Laboratory Strain

It is still the case that molecular, cell, and developmental mycologists use a few strains of their study organism to facilitate comparison of research conducted in different laboratories and at different times. For example, neurosporologists favor *Neurospora crassa* OR74A (also known as FGSC 2489), saccharomycetologists favor S288C, cryptococcologists favor H99, candidologists favor SC5314, aspergillologists favor A4, and so on. Alas, in doing so, they miss out on the alleles and traits found in natural strains and they work with an individual that has become adapted to the laboratory environment and is no longer representative of the wild population from which it was cultivated. The key question for cell and developmental mycologists is, Would moving to multiple wild strains to better associate genetic and phenotypic variation be worth the loss of comparability found in a standard strain?

First, it must be acknowledged that mycologists who work with standard strains go to a lot of effort to develop genetic variation in the standard strain by inducing mutations. With the forward-genetic approach, which begins with a phenotype of interest and artificially induces mutations in thousands of strains, the mycologist then has to screen thousands of mutants and, having found the desired mutant phenotype, associate the mutant gene with the mutant phenotype. If, instead of a phenotype, the researcher begins with an interest in a particular gene, then the process is reversed and the gene is first mutated or deleted, and the search begins for a phenotype, a process that can seem neverending given that a particular phenotype may be encountered only under very specific and unknown conditions. For both forward and reverse genetics, the sequencing of the standard strain is a boon that facilitates either identifying the gene or confirming that it, alone, has been mutated, but it is also a boon that reinforces the community focus on standard strains.

Quantitative Trait Loci

A traditional way of increasing genetic and phenotypic variation in a study without resorting to mutagenesis is to mate the standard strain with a partner that shows genetic and phenotypic differences and then use the progeny to associate phenotypic and genetic variation. As mentioned above, the progeny will display variation in the form of combinations of alleles and resulting phenotypes that exceed those seen in nature. The genes or loci responsible for complex, quantitative traits can then be mapped in this quantitative trait locus (QTL) approach using markers developed to cover the genome. This approach was used with the filamentous fungus *Neurospora crassa* to find QTL associated with female mate choice and to show that the reinforced barriers to sexual reproduction behind mate choice had evolved by natural selection (98). However, as is often the case with QTL studies, the authors were not able to find specific genes responsible for the trait. This problem arises because each QTL can comprise tens to hundreds of genes, owing to the relatively few recombination events in a meiosis, even when hundreds of independent meioses are involved in generating a progeny population. With QTL analysis, full genomes are not a panacea, because it is not a paucity of markers that is the problem but, rather, the large regions in linkage.

Two means of decreasing the size of the linked regions are typically employed. One, an advanced intercross, simply increases the number of matings and meioses used to make the progeny for the mapping population. Although it is labor-intensive to make the cross, this method has been used successfully with *S. cerevisiae* to associate genes with the phenotype of stress resistance (99). The other approach, bulked (or bulk) segregant analysis, increases the number of progeny and, when phenotyping the progeny, sorts those with the most extreme phenotypes into two groups, e.g., those that are the most tolerant and least tolerant to a stressful environment. Bulked segregant analysis is even more efficient when the phenotypic screen recovers only survivors, for example, growth in the presence of an antifungal drug. Individuals with the extreme phenotypes, or the survivors, are sequenced en masse, and the reads are aligned. In these alignments, nearly all genes show the variation present in both parents, except for genes strongly associated with the phenotype; here, the allele of one or the other parent will predominate. Bulked segregant analysis has been used successfully with fungi to find genes involved in sugar metabolism in *Saccharomyces* (100), salt tolerance in the Ascomycota *Lachancia* (101), and, in *Neurospora*, temperature-responsive cell cycle regulation (102) and germling communication and fusion (103).

Recognizing Populations of Fungi

The genetic and phenotypic variation in a study can be further increased by studying many individuals in a wild population and then using an approach developed for studies of human genetics, genome-wide association studies (GWAS), or reverse ecology, an approach developed to mine populations of genomes, among them *S. cerevisiae*, to find genes with a history of strong natural selection (104). As noted above, with QTL analysis, even the hundreds of independent meioses resulting from a cross of fungi cannot break long, linked regions of the genome. In contrast, the old history of mating and recombination in a wild fungal population makes for very short linked regions and remarkable precision in associating genes and phenotype. For example, a recent GWAS in *Neurospora* found that single nucleotide polymorphisms (SNPs) in the 3′ untranslated region of a gene, but not SNPs in the coding region, were associated with the phenotype, a hypothesis validated by subsequent gene deletion experiments (105). GWA requires that populations first be accurately recognized (Fig. 4). If GWA is mistakenly practiced on a mixture of even two populations, the strongest phenotypic differences, which are most likely to be studied, will be between the populations and will associate with every fixed, genetic difference between populations. At the outset, one cannot know the extent of a fungal population, so the genomes of a representative sample of individuals, selected to maximize geographic and phenotypic diversity, must be sequenced, and the variation in the DNA sequence, i.e., the SNPs, compared to identify the populations. Well-diverged populations can be recognized by using the SNPs to construct a phylogenetic tree or network. Population structure can also be discovered using clustering approaches. Phylogenetic trees have the advantage that statistical support for branches can be assessed; clustering methods have the advantage that admixture can be detected. The process of recognizing populations is iterative and may require additional collection if what was thought to be a single, interbreeding population proves to be a complex of several populations.

Genome-Wide Association Studies (GWAS)

GWAS in humans require thousands to tens of thousands of individuals to accurately associate genes with complex traits, e.g., heart disease. However, with fungi, studies employing as few as 23 individuals have found genes associated with traits as complex as pathogenesis or signaling between individuals in a species of fungi, although these genes enjoyed a balance of alleles in the population and a strong effect on the phenotype. A pioneering GWAS of virulence in the Basidiomycota pathogen of coniferous trees, *Heterobasidion*, used just 23 individuals to find a dozen SNPs associated with virulence, and 4 of them were in genes already known to affect virulence in other fungi (106). Cell-to-cell communication and fusion between germinating conidia provided another complex trait where GWAS successfully associated a gene coding for a calcium sensor with the communication trait using just 24 *Neurospora* individuals, albeit 24 individuals preselected from a larger, preliminary screening (105). In this *Neurospora* study, the one significant association, to a calcium sensor, led to another two genes whose proteins were known to associate with the sensor and, when a higher likelihood of false positives was accepted, another six genes associated with the trait. In this study, the nine hypothesized associations were validated (i.e., could not be disproved) by gene deletion experiments (105). Among recent studies with plant-pathogenic Ascomycota (reviewed recently [107]), GWAS found a new virulence factor in *P. nodorum* (108), the gene coding for a small secreted protein virulence factor in *Zymoseptoria tritici* (109), and genes for virulence, toxin production, and sensitivity to azole antifungal drugs in *Fusarium graminearum* (110).

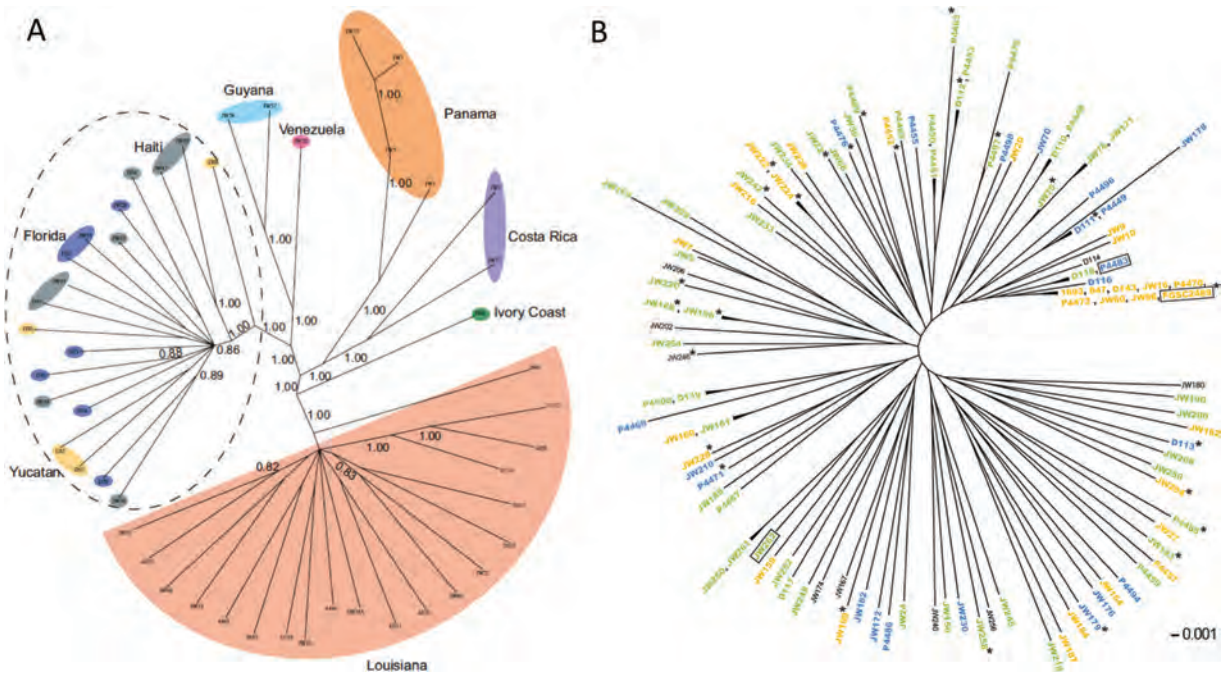


Figure 4 Population structure. (**A**) Bayesian phylogenetic analysis of SNPs from transcriptomes of 50 *Neurospora crassa* individuals from around the Gulf of Mexico showing that individuals thought to form one population actually are found in seven populations. Adapted from reference 112. (**B**) Bayesian phylogenetic analysis of SNPs from transcriptomes of 112 *N. crassa* individuals from the same geographic area as the Louisiana population in A showing no population subdivision. Note the many individuals with the same genotype as the laboratory strain, FGSC 2489, indicative of mistakes made in transferring isolates. Adapted from reference 103.

Hybridization

The ideal natural population for GWAs would be an old, natural hybrid population with twice the variation of the species contributing to the hybrids and the short genomic regions in physical linkage found in freely interbreeding groups. There is evidence that these populations do occur in fungi, as shown by a study of a parasite of wild grass in Iran, *Zymoseptoria pseudotritici*, a relative of the well-known wheat pathogen, *Z. tritici* (111). Comparison of genomes from five *Z. pseudotritici* individuals showed, in half of the genome, twice the genetic variation seen in *Z. tritici* and, in another half, no variation. In the genome half where variation was found, it was of just two types, and within each type there was no variation. The authors inferred that *Z. pseudotritici* originated from a population of F1 hybrids between two, as yet undiscovered, *Zymoseptoria* species, that the hybrids were not able to mate with either parent population, and that severe bottlenecks caused the loss of parental variation in the young F1 population. The example of *Z. pseudotritici* adds homoploid hybrid speciation (speciation originating by hybridization with no increase in chromosome number) to the ways that fungal species can originate and, in this case, it was estimated to have occurred as recently as 200 years or 380 to 550 sexual generations ago. *Z. pseudotritici* sports dispensable chromosomes, like *Z. tritici*, which may indicate that such chromosomes are not restricted to fungi that parasitize agricultural crops. The *Z. pseudotritici* results found hybridization without introgression, but hybridization with introgression has also been discovered in fungi and provides a way for mycologists to discover genes important to adaptation, as will be discussed in the next section.

Gene Flow between Populations

Where population genomic studies of fungi have discovered more than one population or species, they have often also found evidence of gene flow into the populations or among the species. The explanation for this gene flow is that mating between members of the different populations or species leads to hybrids, and these hybrids can then mate with individuals in either of the parental populations, promoting introgression of genes

from one population into the other. The regions that persist in the recipient population are those whose genes confer a selective advantage to the recipient and, if the advantage is strong enough, sweep through the recipient population. These regions can be detected in populations of fungi with sequenced genomes because they show either exceptional divergence or exceptional similarity compared to the surrounding genome, as described below.

When the population divergence is recent and the genomes of both populations are aligned, regions of exceptional divergence are easily recognized against the background of very similar regions (Fig. 5). Where the population divergence is older, regions of exceptional similarity stand out, but regions of exceptional divergence also may be detected (Fig. 6). The genomes resequenced from the populations need not be complete; they can even be transcriptomes, as long as one well-sequenced genome is available to align the others. To search for regions of exceptional divergence or similarity, measures of genetic diversity are calculated for short windows of aligned sequence and the process is repeated as this virtual window is moved along the entire genome. Measures of divergence that have been used for these sliding windows include measures of relative divergence (relative between populations), e.g., Fst, and those of absolute divergence (within regions of aligned genomes), e.g., Dxy (112). Dxy is simply the number of nucleotide substitutions per site between populations and Fst is the proportion of genetic variance found in one population compared to that found in all populations. Use of several measures is recommended because each can be confounded by different patterns of nucleotide variation (113, 114).

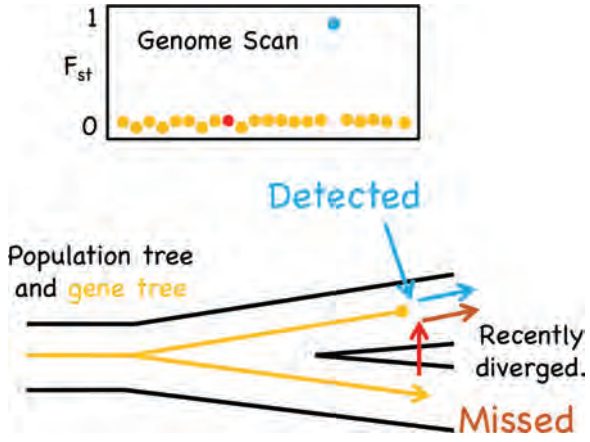


Figure 5 Hybridization and introgression in weakly diverged populations. Hybridization and introgression can be detected in genome scans of closely related populations when the genes are introduced from a more diverged population. (**Top**) Genome scan by Fst (a measure of relative genetic divergence) showing that nearly all genes have low divergence (yellow dots and one red dot), but one gene shows exceptionally large divergence (blue dot). (**Bottom**) Population tree with one gene tree highlighted in yellow showing that well-diverged genes entering from older, more diverged populations (blue dots and arrows) will be detected by comparison with the low divergence in the rest of the genome. However, genes exchanged between the populations will be missed (red dots and arrows) due to their low divergence being indistinguishable from the rest of the genome.

There are limits to this approach. In the case of recently diverged populations, the scant variation between the populations is not sufficient to permit discovery of any regions that have moved between the population, although such exchange must be frequent (Fig. 5). Instead, it is regions that have invaded the populations from more diverged populations, likely unknown, that can be found (Fig. 5). Conversely, when comparing well-diverged populations, regions that move into one or the other population from evolutionarily more distant populations may not appear to be more diverged than the rest of the well-diverged genomes (Fig. 6). In this case, it is the regions that move between the populations that are easily detected, because they show little or no divergence (Fig. 6).

Yeast Population Genomics

As might be expected, the first fungal report of population genomics was with *S. cerevisiae* (115), finding populations in the species that correlated with geography and evidence of hybridization, likely associated with domestication of this socially important fungus. When 99 Asian individuals were added to the analysis, the amount of genetic variation doubled and new populations were discovered, some of them in areas undisturbed by human activity (116). Surprisingly, neither GWAS nor reverse ecology has been practiced with *S. cerevisiae*, although the potential for such studies seems immense (117). One reason that such studies in yeast would be rewarding is that hypothesis testing is possible on a remarkably large scale, as shown in a recent study of sugar use in *S. cerevisiae* and its close relative, *Saccharomyces bayanus* (118). *S. cerevisiae* is a picky eater, preferring glucose when fed mixtures of glucose and galactose, whereas *S. bayanus* consumes both indiscriminately. To test the hypothesis that the phenotype of sugar use was under control of the seven-gene galactose pathway, the researchers swapped promoters of all seven genes, en masse, from *S. cerevisiae* into *S. bayanus*, largely recapitulating in *S. bayanus* the *S. cerevisiae* phenotype. As the authors note, "genetic mapping of complex phenotypes is within reach" (118).

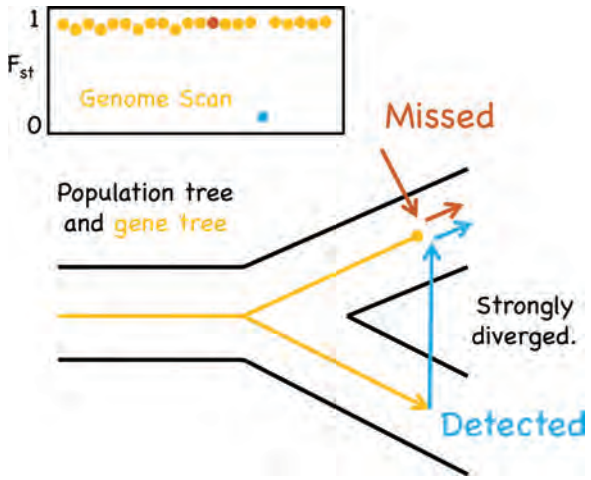


Figure 6 Hybridization and introgression in strongly diverged populations or species. Hybridization and introgression can be detected in genome scans of distantly related populations or species when the gene flow is between the two well-diverged groups. (**Top**) Genome scan by Fst (a measure of relative genetic divergence) showing that nearly all genes have high divergence (yellow dots and one red dot), but one gene shows exceptionally low divergence (blue dot). (**Bottom**) Population tree with one gene tree highlighted in yellow showing that genes exchanged between the populations will be detected (blue dots and arrows) due to their lack of divergence compared to the high divergence of the rest of the genome. However, genes entering from populations from other well-diverged lineages (red dots and arrows) will show divergence similar to the rest of the genome and be missed.

It is *Saccharomyces paradoxus*, the sister species to *S. cerevisiae*, in which population genomics has been researched, enabled by studies of its populations (119) and more than 100 individuals (120). In two North American *S. paradoxus* populations, study of adaptation to the environmental parameters of temperature and frequency of freeze-thaw cycles found that the more northerly population was better adapted than the more southerly one to growth at extreme low and high temperatures and to growth during freeze-thaw cycles (121). The authors attributed the adaptation to a higher mean temperature in the south that resulted in fewer days with temperatures both above and below freezing. Subsequent genome sequencing discovered a third population sympatric with the southerly end of the southern population that originated from hybridization between the northern and southern populations (122). Polyploidy was not involved, as it was with the genome duplication associated with the origin of the *S. cerevisiae* clade; rather, homoploidy was involved, and members of this population have the tolerance for extreme temperatures found in the northern population and the sensitivity to freeze-thaw cycles found in the southern one. Based on genetic similarity, the third population arose by introgression of northern genes into individuals from the original southern population such that the third population has between 2 and 6% northern genes. Interestingly, the third population shows the same reduced fertility in crosses to either parent, perhaps due to reinforced barriers to mating that can arise in sympatry, as was noted above for *Neurospo*ra (98).

Reverse Ecology: Ascomycota Populations

Among filamentous Ascomycota, *N. crassa* has been a model for population studies due to a remarkable collection of wild isolates (123) and a well-studied laboratory strain that has been the subject of both a genome sequencing project (2) and a comprehensive gene deletion project (124). An *N. crassa* population bordering the Gulf of Mexico that included the laboratory strain was chosen for GWA (125). When 50 of the individuals had been sequenced, phylogenetic analysis revealed not one but seven populations (112) (Fig. 4). No population had enough individuals for a GWAS, but fortunately, two of them had at least 20 members, one in Louisiana and one further south in the Caribbean. These two populations differ by 2° to 10° of latitude and a difference in average winter temperature of 9°C. When the aligned genomes were scanned to detect regions of exceptional divergence, using measures of both relative and absolute divergence, more than 30 such regions qualified, but only 2 were identified by all three approaches. Among the genes in these two regions were two known to protect against cold temperatures: a cold shock RNA helicase and a prefoldin chaperone that, in yeast, protects actin from cold temperatures (Fig. 7). The resulting hypothesis, that the Louisiana population had adapted to life at low temperature, was not disproved by measuring the growth of 10 isolates from each population at low (10°C) and normal (25°C) temperature.

A second test was devised using a similar growth approach and the aforementioned gene deletion collection (124). For all eight genes found in the two regions, a gene deletion strain was obtained from the gene deletion collection, and these strains were screened at 10°C. Most gene deletion strains showed no loss of cold tolerance (an important control for the possibility that simply deleting genes would affect cold tolerance), but this was not so for strains deleted for the RNA helicase or the prefoldin. The same result was again found in progeny of crosses between these two deletion strains and wild type; i.e., inheritance of the wild-type allele conferred adaptation to low temperature that was lost if it was deleted. The authors called this approach to dis-

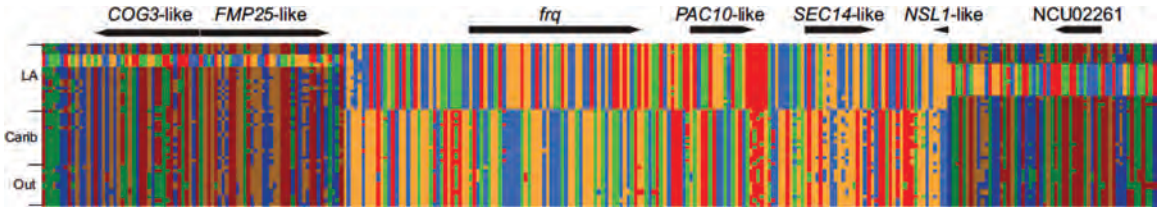


Figure 7 Regions of extreme divergence between populations of *N. crassa*. Rows are aligned genomes of Louisiana (LA), Caribbean (Carib), and other populations (out) seen in Fig. 4. Columns are nucleotide positions in four colors for the four bases. Highlighted is the region of high divergence between the Louisiana population and the Caribbean and other populations. The genome variation in this region is consistent with a history in the Louisiana population of hybridization and introgression. Low variation among Louisiana individuals in this region is consistent with a recent selective sweep. Variation in the length of introgressed regions in the Louisiana population may indicate that the sweep is still in progress. Among the six genes in the region of divergence is *PAC10*-like, which codes for a prefoldin that chaperones cold-sensitive proteins. Adapted from reference 112.

covering adaptation reverse ecology (104), because it is the reverse of most studies of adaptation. Normally, adaptation studies begin with knowledge of a phenotype adapted to an environmental pressure, e.g., the darkening of coat color in mice that have adapted from life on light-colored desert soils to life on dark-colored lava flows (126). In reverse ecology, one begins with genomes, identifies genes subject to natural selection (in the case of *N. crassa*, selection associated with hybridization, introgression, and selective sweeps), and with the knowledge of the genes and their function combined with knowledge of variation in the physical environment, infers the environmental factor (with *N. crassa*, growth at low temperature).

Reverse Ecology: Basidiomycota Populations

Basidiomycota have also been the subject of reverse ecology, e.g., the dikaryotic, ectomycorrhizal mushroom *Suillus brevipes* (127). This basidiomycete is symbiotic with pines, and 11 individuals were cultivated from fruiting bodies found in coastal California and another 17 from fruiting bodies in the Sierra Nevada of California. Using a guide sequence from the montane population, the 28 genomes were aligned, and half of the 1.2 million SNPs were used to make a phylogeny that found two populations, one coastal and one montane. These two populations are extremely similar, with only 0.01% of the SNPs being reciprocally fixed in the two populations. The same populations were recognized by a population structure approach (128), which showed no admixture. Despite the extremely similar genomes, Bayesian population analysis (129) estimated that the two populations had been diverged for 25,000 generations, with undetectable gene flow. Scans of the aligned sequences using metrics of relative and absolute divergence found two regions of the genome that, together, contained half of all reciprocally fixed SNPs. Of the four genes found in these two regions, one (*Nha1-like*) is invariable and homozygous in the coastal population, suggestive of a hard, selective sweep. It encodes a protein homologous to a Na+/H+ exchange protein found in yeast membranes that is involved with salt tolerance. It is tempting to speculate that the coastal population must contend with more salt than the montane population, but this speculation has not yet been tested. More recently, the *Suillus* study was expanded to include individuals from throughout western North America. With more geographic coverage, the study found evidence of admixture in Canadian populations situated between two, less diverse populations, one in Colorado and the other in Minnesota (130). The authors used evidence for selective sweeps and analyses associating genetic variation with environmental parameters (temperature and moisture) to identify proteins that may be involved in adaptation, e.g., membrane transporters, nucleic acid helicases, and the aforementioned Na+/H+ exchange protein. As often is the case with nonmodel fungi, hypotheses about the involvement of these genes in adaptation await testing.

Reverse Ecology: Ascomycota Species

Our discussion of fungal reverse ecology began with closely related, fungal populations, but the first such study was with well-diverged species in the Ascomycota human pathogens *C. immitis* and *C. posadasii*, agents of the human disease coccidioidomycosis (131). These fungi were studied just as high throughput sequencing was becoming possible, and most of the genomes were sequenced by the traditional Sanger method. The substantial investment was made possible

by prior studies of species and populations of these fungi, which identified at least five populations spread over two species (132, 133), a concrete example showing that evolutionary studies can have an impact in medicine. With the aim of comparing at least two individuals from each population, 20 genome sequences were obtained and aligned. Between the two species, 43% of all SNPs were reciprocally fixed, a much higher proportion than the 9% fixed differences between the Louisiana and Caribbean *N. crassa* populations or the 0.01% between the coastal and montane *Suillus* populations. As with *Neurospora* and *Suillus*, regions of the *Coccidioides* genomes were found with exceptional measures of relative divergence, in this case, exceptionally low Fst, which indicated that as much as 7% of the genome of each species had a history of hybridization and introgression between the two species (131). One region of introgression from *C. posadasii* into the Southern California population of *C. immitis* was amplified and sequenced from an additional six individuals. When aligned, the introgressed regions in all individuals shared a common edge marked by a metalloproteinase gene, Mep4, which is related to a known virulence factor, Mep1 (134). The assumption that selection for Mep4 drove the introgression was challenged when a subsequent study failed to find a difference in Mep4 transcription in the saprobic and parasitic *Coccidioides* growth phases, especially when the study found a 20-fold difference in a small (99 amino acid) previously undiscovered gene lying between Mep4 and the edge of the introgressed region (135). Again, the hypothesis that this small gene drove introgression awaits testing.

Reverse Ecology: Basidiomycota Species

A more recent example of reverse ecology with well-diverged populations or species comes from the Basidiomycota *Cryptococcus gattii*, a haploid capable of causing disease in otherwise healthy humans (136). This example is interesting because it shows that it is possible to find regions of exceptionally large divergence even between well-diverged populations or species if one uses a comparator with a broad scale, e.g., Dxy, rather than one with a restricted scale such as Fst. *C. gattii* has four distinct, species-level (137) groups, VGI-VGIV, with VGII containing the strains responsible for outbreaks of cryptococcosis in otherwise healthy hosts in Vancouver, Canada, and the U.S. Pacific Northwest. Genomes were sequenced from 53 individuals representing all four VGs, with a focus on the outbreak population. From a Dxy sliding-window scan of the aligned VGII sequences, two adjacent regions were found within a 15-kb region of one scaffold that showed exceptionally high percentages of polymorphic sites (136). Phylogenetic analyses of the first of these regions suggested that it originated by hybridization and introgression from a different VG, VGI. In one of the VGII individuals, an introgressed region is also aneuploid and homozygous, suggesting duplication after introgression. This introgressed region contains three genes that, by homology with *S. cerevisiae*, appear to be involved in actin polymerization, dissociation of Rad51 D-loops, and metal ion transport plus vacuole protein targeting. It will be very interesting to see if deletions or allele swaps of any of these three genes have an effect on virulence, although the adaptation implied by the introgression could be related to any biological or physical environmental parameter. It seems likely that the hybridization occurred by sexual reproduction, consistent with the discovery of a diverse, outbreeding population of VGII in South America that also embraces the genetic variation displayed by strains found in northwestern North America, Australasia, and Southeast Asia (136, 138, 139).

Reverse Ecology with Uncultivatable Fungi

Earlier, it was noted that population genomic studies are facilitated when the fungal individuals can be cultivated. A recent study of an obligate plant parasite, one that has never been cultivated, shows that cultivation is not essential. Comparison of genomes of 46 individuals of the obligate plant pathogen that causes powdery mildew, *Blumeria graminis*, that had been collected from three crops—wheat (*B. graminis* f. sp. *tritici*), rye (*B. graminis* f. sp. *secalis*), and the hybrid plant triticale (*B. graminis* f. sp. *triticale*)—revealed a fascinating evolutionary history (140) that recalls the aforementioned study of Zymoseptoria (111). Based on genome comparisons, *B. graminis* f. sp. *triticale* is a recent hybrid of the other two powdery mildews, and one that arose in the past 50 years, coincident with the introduction of the hybrid plant, triticale, to agriculture. *B. graminis* f. sp. *triticale* has regions of DNA from both parents, but most came from *B. graminis* f. sp. *tritici*, indicating that the initial hybridization was followed by introgression of the F1 into the *B. graminis* f. sp. *tritici* population. Based on the amount of genetic variation seen in *B. graminis* f. sp. *triticale*, hybridization involving several parents as well as their F1 progeny likely contributed to its origin. Given that one parent, *B. graminis* f. sp. *secalis*, can cause some disease symptoms on triticale, whereas the other parent, *B. graminis* f. sp. *tritici*, cannot, it is assumed that *B. graminis* f. sp. *secalis* effectors are important to the

ability of *B. graminis* f. sp. *triticale* to infect triticale. Unexpectedly, transcription studies have not implicated any of the known *B. graminis* f. sp. *secalis* effectors found in *B. graminis* f. sp. *triticale*. Again, this is a system with great potential for gene disruption studies to identify the genetic basis of *B. graminis* f. sp. *triticale* infection of triticale.

Hybridization: Caught in the Act

The population genomic studies presented so far show evidence of hybridization and introgression, but none have caught populations in the act of hybridization. The most recent hybrid found so far comes from a recent study of *Neurospora discreta* (141), a close relative of *N. crassa*. One phylogenetic species in the *N. discreta* complex, PS4, has an exceptionally large latitudinal range in North America, from New Mexico to Alaska, and also is found in Europe and Asia. When genomes were sequenced and analyzed for more than 50 individuals from this species, they showed three, well-diverged lineages (with 35 to 45% of SNPs reciprocally fixed between populations): Alaska and Europe (AK-EU), California and Washington state (CA-WA), and New Mexico and Washington state (NM-WA). Among the Alaskan individuals was one possessing, evenly distributed through its chromosomes, 12% of the genome of the apparently allopatric, NM-WA lineage (Fig. 8). Mysteriously, at one collecting site in Washington, where the CA-WA and NM-WA lineages are clearly sympatric, there was no evidence of hybridization. Clearly, this is a case where more individuals should be sampled and studied.

LAST WORD

Every technical advance in examining fungal genomes has added to our knowledge of the type and extent of genetic variation available to fungi. There is every reason to think that this trend will continue as fully assembled genomes of more and more individuals become available to mycologists, for example, the aforementioned population genomic studies of genome rearrangement and epigenetics that are expected to emerge from genomes obtained from single molecule, real-time sequencing.

Among the many outputs of genomic research, none outstrips hypothesis generation. An outcome of this abundance is a bottleneck for the completion of any

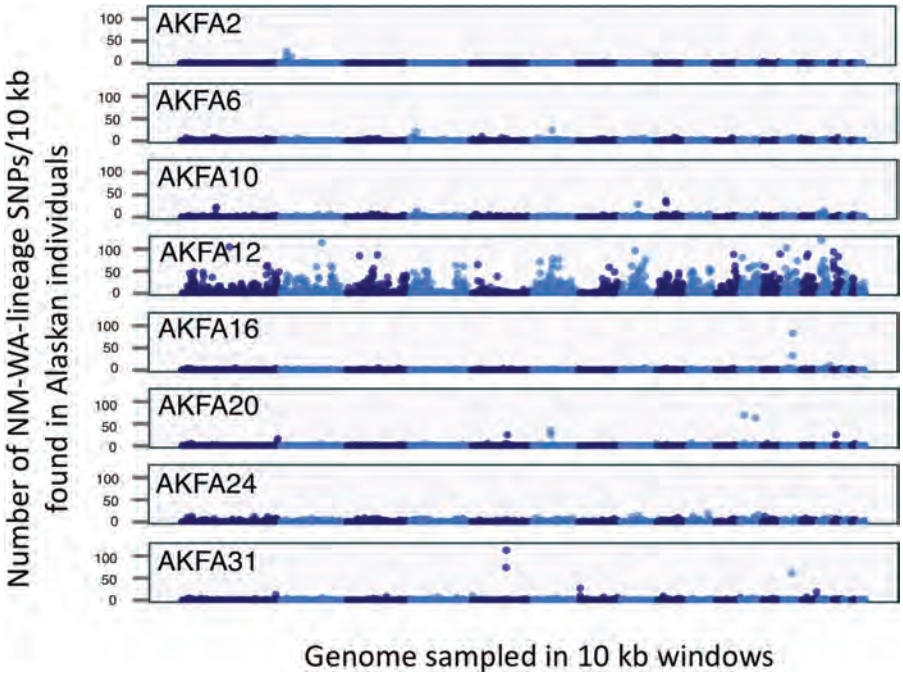


Figure 8 Evidence of recent hybridization. Genome scans for introgressed DNA in the 20 largest contigs of eight *Neurospora discreta* individuals from the Alaska-European lineage. Numbers of SNPs introgressed from the New Mexico-Washington (NM-WA) lineage are shown on the *y* axis. Alaskan strain AKFA12 stands out as having 12% of its genome introgressed from the NM-WA lineage, as expected from a few matings between a hybrid individual and members of the Alaskan population. Adapted from reference 141.

scientific endeavor: hypothesis testing. Here, again, technological advances may prove important. For example, the successful use of CRISPR-associated protein-9 nuclease to transform *S. cerevisiae* (142), filamentous fungi in the Ascomycota genera, *Aspergillus* (143), *Trichoderma* (144), and *Pyricularia* (= *Magnaporthe*) (145), as well as the Basidiomycota genera *Ustilago* (146) and *Cryptococcus* (147), indicates that hypothesis testing in all types of fungi may become, if not routine, then at least possible.

A unifying feature of genetic diversity in fungal genomes, with the exception of HGT between phylogenetically distant lineages, is that every means of generating diversity is operating all the time, such that fungal genomes are remarkably fluid and variable. That there is any constancy at all to fungal genomes is a strong testament to the power of purifying natural selection. Is there something special about fungi in terms of their life cycles or population sizes that makes them unusual in terms of generating genetic variation and enduring particularly strong natural selection? Or is it simply that the data exist for fungi because they threaten our health and food supply? Other Eukaryotic microbes have life cycle features and population sizes similar to those of fungi, as do some animals, e.g., corals. It may be that fungi are not unique in terms of genetic diversity and strength of natural selection and that the same observation will be made for plants and animals when population genomics becomes affordable for these kingdoms.

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RNA Interference in Fungi: Retention and Loss

31

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INTRODUCTION

RNA interference (RNAi) is a mechanism conserved in eukaryotes that represses gene expression by means of small noncoding RNAs (sRNAs) of about 20 to 30 nucleotides (nt). Before the identification of the RNAi mechanism, its effects were observed in different organisms and described as independent processes. These effects were first observed in plants, in which the introduction of sequences intended to increase the production of floral pigments had the opposite effects, resulting in albino transformants. Hence, this phenomenon was called cosuppression (1, 2). The same phenomenon was also observed in the fungus *Neurospora crassa*, in which an albino phenotype was obtained after transformation with extra copies of the gene *al-1*, which is involved in the production of carotenoids (3). The characterization of this phenomenon revealed reversibility of the albino phenotype, since some descendants of the original albino transformants reverted to the wild type phenotype. At that time, this phenomenon was not linked to the process of cosuppression observed in plants and was named "quelling." The discovery of quelling in *N. crassa* represented a milestone in the field of RNAi, because this fungal model allowed the use of classic genetic tools to unravel the machinery of RNAi. The molecular mechanism of RNAi was finally uncovered by Fire et al., who discovered the central role of double-stranded RNA (dsRNA) in the RNAi of *Caenorhabditis elegans* (4). This central role of dsRNA was soon established in all the organisms harboring a functional RNAi mechanism, including plants, fungi, and animals. Cosuppression, quelling, and other posttranscriptional gene-silencing-related phenomena were integrated into the same conserved mechanism of RNA interference (5).

The initial function assigned to RNAi was its role as a defensive mechanism against invasive nucleic acids such as viruses, transposons, and other exogenous sequences such as transgenes and plasmids (6). The protective mechanism of RNAi is based on targeting and repression of the transcription (transcriptional gene silencing) or translation (posttranscriptional gene silencing [PTGS]) of invasive nucleic acids. However, the endogenous regulatory function of this mechanism is the aspect of RNAi that has attracted most of the research in this field. In plants and animals, the discovery of microRNAs (miRNAs), small endogenous RNAs that control the expression of target genes, unveiled the role of RNAi as a whole new regulatory layer at the RNA level, controlling most of the known cellular processes such as growth, development, differentiation, cell death, and disease (7, 8). In fungi, the regulatory role of RNAi has been extensively studied in the fungus *Mucor circinelloides*, as RNAi mutants in this fungus are affected in growth, hyphal morphology, sexual and asexual sporulation, stress response, and cell death (9–11). No miRNAs have been found in *M. circinelloides*, although particular regulatory small RNAs called exonic-siRNAs (ex-siRNAs) were identified and characterized in this fungus (12).

The RNAi mechanism is highly conserved in the fungal kingdom (13), which together with its defensive and regulatory roles, suggests that it is an essential mechanism that has been positively selected during evolution. However, the discovery of several fungi lacking an active RNAi pathway raises the question of how they can survive without the protective and regulatory role of RNAi. This article will discuss this question and will also review the machinery, mechanism, and functions of RNAi.

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COMPONENTS OF THE RNAi MACHINERY

The machinery of RNAi is well conserved in the eukaryotic domain, although different functional pathways can be found in different groups of organisms or even in the same organism. In the fungal kingdom, these pathways involve three specialized components that comprise the central core of the fungal RNAi machinery: an RNA-dependent RNA polymerase (RdRP), the enzyme Dicer, and the Argonaute (AGO) protein (Fig. 1). The activation of the mechanism by sense transgenes depends on the RdRP component, which produces dsRNA molecules from single-stranded RNA precursors. Then the second component, an RNase III ribonuclease known as Dicer, cleaves the dsRNA and generates short interfering RNAs (siRNAs). These small RNAs are loaded into the third component, an AGO protein that usually forms a complex known as RISC (RNA-induced silencing complex), which uses the siRNA as a guide to target complementary specific RNA sequences. The interaction of AGO-containing complexes with an RNA target may block either transcription or translation or induce the degradation of the target RNA. Fungi often have more than one gene for each of the three basic RNAi components (13), and it is also common to find different functions for each member. Besides these three basic components, there are other pathway-specific components that are also reviewed in the corresponding sections.

The RdRP

RdRP enzymes can be found in plants, nematodes, and most fungi, except basal fungi such as the chytrid *Batrachochytrium dendrobatidis* and the microsporidia *Nosema ceranae* (13). Recent studies place RdRPs right at the onset of RNAi activation, because they have been proposed to directly produce aberrant RNA (aRNA) from a DNA template. The crystal structure of QDE-1, the RdRP of *N. crassa* involved in quelling, revealed a strong similarity to eukaryotic DNA-dependent RNA polymerases, instead of viral RdRPs, suggesting that QDE-1 might act as both an RdRP and a DNA-dependent RNA polymerase (14,

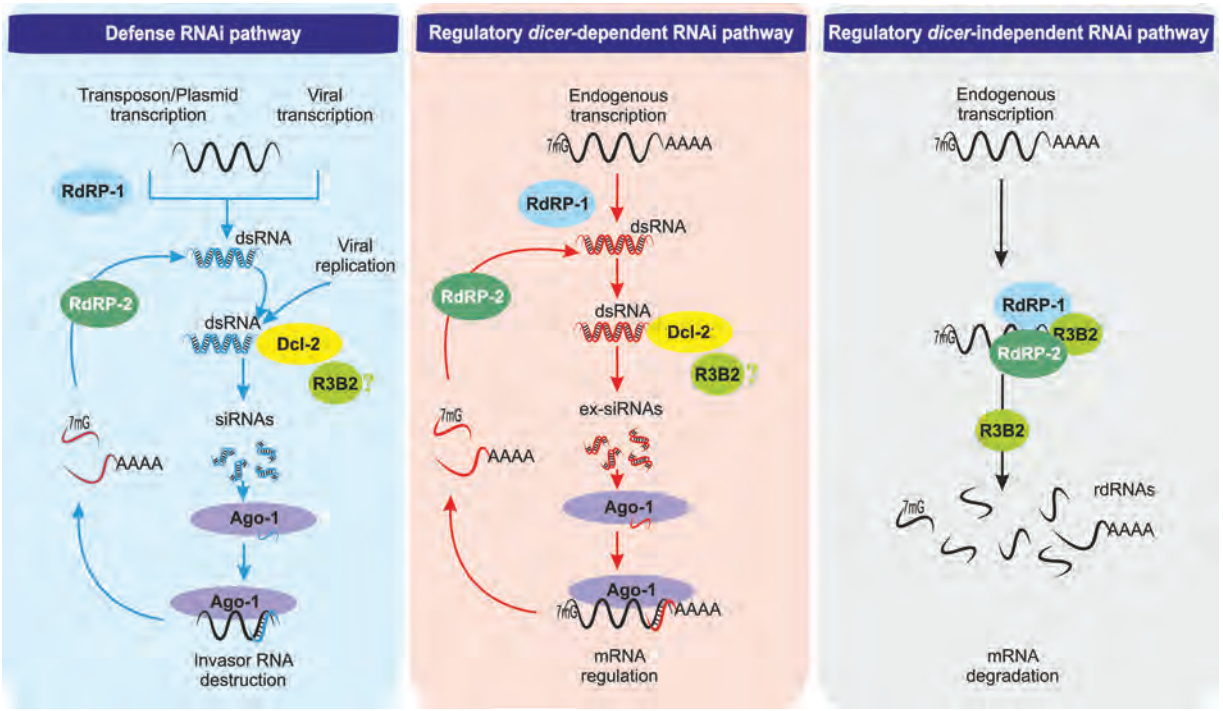


Figure 1 Main RNA interference (RNAi) pathways identified in *Mucor circinelloides*. Fungal RNAi-mediated defense mechanisms against exogenous nucleic acids in fungi is exemplified by the *M. circinelloides* defense mechanism (left box). This fungus shows an amplification step mediated by RdRP-2, which has not been found in other fungi. In addition to this defense pathway, this fungus shows two distinct RNAi pathways to regulate the expression of endogenous genes (central and right boxes). Question marks indicate that the R3B2 protein participates in these pathways, although its precise function is unknown.

15). These two activities of QDE-1 were experimentally demonstrated, confirming that RdRPs could be required both for the production of the aRNA and the subsequent dsRNA generation (14). The most intriguing question about the mechanism of RNAi activation is how production of aRNA occurs in specific DNA sequences, e.g., transposon sequences. This question has been recently answered in a study that links double-strand breaks in DNA and homologous recombination with the production of aRNA and further RNAi activation (16). Repetitive DNA, one condition known to be necessary for RNAi activation in *N. crassa*, is susceptible to DNA damage, which in turn is the signal for QDE-1 to synthesize aRNA from the affected region. The second step in the activation of RNAi is the synthesis of dsRNA, which occurs via RdRP enzymes that transform the aRNAs into the triggering dsRNA molecules (17, 18).

In organisms that show amplification of the silencing signal, RdRPs have a third function devoted to stabilizing silencing through the production of secondary siRNAs (19, 20). Secondary siRNAs are produced by RdRPs from the targeted RNAs rather than from the initial triggering molecules like primary siRNAs. Secondary siRNAs can be produced upstream and downstream of the initial inducer sequence, whereas primary siRNAs are only generated from sequences of the initial triggering dsRNA molecules. This is a process known as "transitivity," and it can be different depending on the organism in which it has been studied. In fungi, this phenomenon has been described only in *M. circinelloides* (Fig. 1), which has three RdRPs. Two of these RdRPs, RdRP-1 and RdRP-2, are involved in RNAi with differentiated roles for the initiation and amplification steps. RdRP-1 is involved in the activation of RNAi by the synthesis of dsRNA from the sense transgene, showing no activity related to the accumulation of secondary siRNAs or silencing stability (21). Therefore, the function of RdRP-1 in *M. circinelloides* seems to be similar to the QDE-1 of *N. crassa* in RNAi induced by sense transgenes (17, 22). Conversely, RdRP-2 of *M. circinelloides* only participates in the amplification step of silencing. Thus, an *rdrp-2* mutant shows a low silencing frequency and very unstable silenced phenotype both in RNAi induced by sense transgenes and in dsRNA producing transgenes (21).

The Dicer Enzyme

The main characteristic of Dicer enzymes is their ability to cleave dsRNA molecules (Fig. 1), an enzymatic activity that defines the RNase III family of ribonucleases. The way of cleaving dsRNA is also specific to this family, which consists of leaving the 5′ end with a monophosphate group and 2-nt overhang at the 3′ end (23). Dicer enzymes are only found in eukaryotes, displaying a unique structure based on two tandemly arranged RNase III domains, a single dsRNA-binding domain, an amino-terminal helicase DEXD/H-box domain, a small domain for protein-protein interaction (the DUF283 domain), and a PAZ (PIWI-Ago-Zwille) domain. The two catalytic domains are arranged to adopt an intramolecular homodimerization in charge of cleaving the two strands of dsRNA, resulting in the production of short dsRNAs of 21 to 25 nt (24). The length of the product is determined by the distance between the PAZ domain and the RNase III domains, which can change in different organisms (25). This distance is determined by a long nonconserved helix that functions as a molecular ruler and explains the different sizes of siRNAs found across species (26).

The fungi that have, or likely have, RNAi pathways often possess more than one gene coding for the Dicer enzyme (13). *N. crassa* has two *dicer* genes, *dcl-1* and *dcl-2*, which have redundant functions (27), whereas the two Dicer enzymes found in *M. circinelloides*, Dcl-1 and Dcl-2, present distinct functions, because only Dcl-2 is required for a functional RNAi mechanism (28). *M. circinelloides* mutants in the *dcl-1* gene show a fully functional RNAi mechanism, indicating that this gene is not essential for RNAi induced by invasive nucleic acids. Interestingly, these mutants presented an abnormal phenotype with reduced growth and altered hyphal morphology. These observations were the first evidence involving the fungal RNAi mechanism in the endogenous regulation of cellular processes (10).

The AGO Protein

The AGO protein is the third essential component of the RNAi machinery. Its role is to load the guide strand of the siRNAs generated by Dicer in the AGO-containing complexes, named RISC (Fig. 1). AGO proteins harbor a specific binding pocket in which the sRNA guide strand is anchored. The AGO-containing complexes use this guide strand to target the corresponding complementary sequence, which is either transcriptional or translationally repressed. In the case of PTGS, the degree of complementarity between the guide sRNA and the target determines either a slicing event and further release of the target, in case of perfect complementary (29, 30), or translation blockage of the target mRNAs and prevention of RISC complex release, in the case of partial complementary. Eventually, inhibition of translation leads to degradation of the target mRNA (31).

The architecture of the AGO protein family is based on three well-conserved domains: PAZ, MID (middle), and PIWI (32, 33). The guide siRNA binding pocket is harbored in the PAZ domain, which anchors the two overhang nucleotides of the 3′ end, whereas the MID domain binds the 5′ phosphate at the other end (34, 35). The PIWI domains contain the catalytic activity of the AGO proteins, which relies on a catalytic center similar to RNase H. This catalytic domain possesses a triad DDH, with the two aspartate residues being highly conserved (32, 33). All fungal AGO proteins share a well-conserved domain architecture, although functional diversification is observed in different fungi. For example, the AGO protein of *N. crassa*, named QDE-2, and Ago-1 of *M. circinelloides* have an essential role in RNAi induced by exogenous invasive nucleic acids, both with sense RNA- and dsRNA-expressing transgenes (9, 36). Besides an essential role in the induction of RNAi by exogenous sequences, the *qde-2* mutants have no other physiological process affected. However, a second AGO protein of *N. crassa*, named SMS-2, is involved exclusively in meiotic silencing by unpaired DNA (MSUD) (37).

FUNCTIONAL DIVERSITY OF RNAi

RNAi is widely conserved in eukaryotes, including fungi, indicating that this regulatory mechanism evolved before fungi diverged from animals. Thus, the common fungal ancestor possessed a functional RNAi pathway that has evolved along fungal evolution to generate diverse RNAi pathways, which can carry out different functions in extant fungal species. This section summarizes the main functions of RNAi in fungi.

Maintenance of Genome Integrity

In nature, organisms are confronted with a variety of threats, such as viral infections or genome damage, which put their existence at risk. Genome integrity can be affected by the movement of mobile genetic elements or accidental breaks produced spontaneously or induced by chemical or physical mutagens. The persistence of organisms on our planet is possible because they have developed mechanisms to maintain genomic integrity. The RNAi pathways play a predominant role in protecting cells from exogenous genetic material such as viruses or transposons or any other genetic material introduced by any means, as was demonstrated by the experimental introduction of transgenes or plasmids. Some of these protective RNAi mechanisms work specifically during vegetative growth, whereas others operate in processes related to sexual reproduction.

Recent results derived from the characterization of several RNAi pathways have also shown that RNAi is implicated in double-strand break repair in fungi, as occurs in plants and animals (33, 38). Moreover, RNAi also protects genomic integrity by maintaining a repressive heterochromatic state in repetitive regions of the genome, most notably those regions which harbor mobile genetic elements (39).

The next sections focus on the role of RNAi in the maintenance of fungal genome integrity, although several of the conclusions derived from these studies could be applied to other eukaryotes.

Defense responses during vegetative growth

RNAi-mediated defense mechanisms against exogenous nucleic acids that act during vegetative growth can be found in many fungi, and some of them have been characterized in detail. Although the best-characterized mechanism is quelling, in the ascomycete *N. crassa*, other mechanisms have been analyzed in other fungal groups, such as mitotic-induced silencing in the basidiomycete *Cryptococcus neoformans* and gene silencing in the *Mucoromycotina M. circinelloides* (Fig. 1). Mitotic-induced silencing is very similar to quelling in *N. crassa*, because both processes are associated with multiple copies of the integrated transgene, the strength of silencing correlating with transgene copy number (16, 40, 41). In *M. circinelloides*, transgenes do not integrate into the genome because they are carried by self-replicative plasmids, but there is also a correlation between silencing efficiency and transgene copy number (42). All of these silencing mechanisms act posttranscriptionally and require core RNAi components and accessory proteins. In organisms in which more than one protein for each core component is present, a functional specialization is usually observed. However, this is not the case for Dicer enzymes in quelling, because both proteins are redundant. In addition to the two Dicer enzymes, quelling in *N. crassa* requires one particular AGO protein (QDE-2), one particular RdRP (QDE-1), and the exonuclease QIP (QDE-2-interacting protein), which cleaves and removes the nicked passenger strand from the siRNA duplex in a QDE-2-dependent manner (17, 43). Mitotic-induced silencing in *C. neoformans* only requires one of two Dicer proteins (Dcr2) in addition to the single AGO and RdRP proteins (41, 44), while transgene-induced PTGS in *M. circinelloides* requires one of the two Dicer proteins (Dcl-2), one particular AGO protein (Ago-1), QIP (our unpublished data), and two RdRPs, which carry distinct functions (45).

The initial molecular events that trigger siRNA production and subsequent PTGS have been elegantly

deciphered in *N. crassa* and connected with maintenance of genome stability (16). In this fungus, quelling is tightly associated with the presence of multiple transgene copies, particularly tandem repeats, rather than integration at multiple sites. However, the tandem repeats alone are not sufficient for gene silencing and siRNA production; double-strand breaks at the tandem repeat locus and homologous recombination (HR) are also required (16, 46, 47). Quelling is initiated when a double-strand break occurs in the tandem repeat locus that results in HR of repetitive sequences. The model proposes that recombination intermediates produced during HR can be specifically recognized by components of the quelling pathway, likely QDE-3, a putative RecQ DNA helicase (48). QDE-3 may resolve the recombination intermediates into ssDNA using its DNA helicase activity. It also recruits the replication protein A (RPA), a single-stranded DNA-binding complex, and the dual-functional enzyme QDE-1, which may use its DNA-dependent RNA polymerase and RdRP activities to first produce aberrant single-stranded RNA and then dsRNA to activate the downstream RNAi pathway (14). This mechanism ensures that siRNAs are specifically produced from repetitive DNA loci, such as those corresponding to transposons, but not from nonrepetitive regions of the genome.

In addition to PTGS, the RNAi pathway is involved in maintaining genome integrity because mutants that lack a functional RNAi pathway cannot maintain transgene tandem repeats after double-strand breaks, showing a rapid loss of the repeats (16) and reduced copy number of the highly repetitive sequences of rDNA loci (49). Moreover, DNA damage in *N. crassa* results in the production of rDNA loci-derived QDE-2-associated sRNAs, named qiRNAs, that require the same components as quelling (QDE-1, QDE-3, Dicer, and RPA) (14, 50) and HR (46, 47). Repetitive sequences are known to be a major source of genome instability due to hyperrecombination events as a result of replication stress (51, 52). Moreover, the Dicer enzyme (Dcr1) of *Schizosaccharomyces pombe*, but not other components of the RNAi pathway, promotes the release of RNA polymerase II (Pol II) from sites of replication stress and DNA damage, such as rDNA loci, where Pol II release facilitates DNA replication and prevents homologous recombination, which would otherwise lead to a loss of rDNA repeats, especially during meiosis (53). DNA damage-induced small RNAs have been reported in other eukaryotes such as *Arabidopsis*, *Drosophila*, and mammals (9, 33), suggesting that DNA damage is a common trigger for sRNA production in eukaryotes. In summary, small RNA and RNAi components might have a broad role in maintaining genome stability in eukaryotes, including defense against transposons.

Fungal RNAi pathways are supposed to protect against fungal virus (mycovirus), because most known mycoviruses have dsRNA or single-stranded RNA genomes (54). Although mycoviruses are widespread in all major taxa of fungi (55), their control by RNAi has been demonstrated only in the ascomycete *Cryphonectria parasitica* (56). In this fungus, specialization of the RNAi core components also is remarkable because only one of two Dicer enzymes (DCL2) and one of four AGO proteins (AGL2), but none of four RdRPs, are involved in defense against several mycoviruses (56–58). Interestingly, some mycoviruses have developed strategies for interfering with this antiviral RNAi-mediated silencing for their survival (59). Importantly, the characterization of these strategies can contribute to understanding the RNAi machinery itself.

Sex-induced silencing

The RNAi-mediated defense mechanisms that work in vegetative mitotic growth have in common that they are triggered for multiple copies of a DNA sequence, which are integrated either in tandem in ascomycetes and basidiomycetes or in self-replicative plasmids in the *Mucoromycotina M. circinelloides*. Interestingly, in the basidiomycete *C. neoformans*, a transgene-induced posttranscriptional silencing mechanism silences tandem transgene arrays at a high frequency during the sexual cycle but at a frequency ~250-fold lower during asexual mitotic vegetative growth. Hence, this phenomenon was named sex-induced silencing (60), and it operates at similar efficiency during both unisexual reproduction and opposite-sex mating (61). Sex-induced silencing is mediated by RNAi via sequence-specific sRNAs and requires central components of the RNAi pathway, including the single AGO protein, the two Dicer enzymes, although Dcr2 plays the major role, and the single RdRP (60). The small RNAs map to repetitive transposable elements, suggesting that the main function of sex-induced silencing is to protect genome integrity from the activity of selfish DNA elements during mating, reducing the generation of a high mutational burden. This may be crucial for *C. neoformans* because transposons and retrotransposons are more active during sexual reproduction. According to this hypothesis, RNA machinery components are more abundant during mating or under mating growth conditions (60).

Meiotic silencing by unpaired DNA

Studies of *N. crassa* have revealed the existence of an RNAi-related mechanism that protects genome integrity

during meiosis. This mechanism transiently silences any sequence that is unpaired during meiosis, so it was called meiotic silencing by unpaired DNA (MSUD) or simply meiotic silencing (62). This process is initiated by the identification of the unpaired sequences in the prophase I of meiosis I and lasts until ascospores are formed (63). MSUD is mediated by small RNAs, referred to as MSUD-associated small interfering RNAs (masiRNAs), which are thought to direct silencing of mRNAs by complementary base pairing. masiRNAs are similar to siRNAs generated in quelling, but not identical, since they are mostly 25 nt in length and have a strong bias for 5′ uracil (64).

MSUD can be divided into two main distinct processes: detection of unpaired DNA and silencing of the corresponding sequences. The mechanism for detection of unpaired regions is poorly characterized, although two essential proteins for MUSD, SAD-5, and SAD-6 may be involved in this process because of their nuclear localization. Interestingly, SAD-6 possesses a well-characterized domain that places it within a protein family that includes *Saccharomyces cerevisiae* Rad54. Enzymes from this family participate in searching for DNA homology during double-strand break repair by homologous recombination, suggesting that identification of unpaired DNA in MUSD may use a similar mechanism (65). Unpaired DNA detection is thought to trigger the production of an aRNA that could be delivered to a silencing complex stationed in the perinuclear region, where silencing occurs (66). In this region seven proteins are located that are essential for MUSD, and at least six of them form a complex, which includes several RNAi proteins, some of them shared with quelling (66). There, the aRNA is converted to dsRNA by the RdRP SAD-1, with the participation of the helicase SAD-3. Then, Dicer DCL-1 processes the dsRNA into masiRNAs, which are bound to the AGO SMS-2, and the passenger strand is removed by the exonuclease QIP. MasiRNAs bound to SMS-2 are utilized to target homologous mRNAs to posttranscriptional silence of unpaired DNA and any other homologous sequence (37). The complex is maintained by SAD-2, a presumptive scaffold protein that tethers these silencing factors in the perinuclear region. In addition, a seventh protein located in the nuclear periphery, SAD-4, is required for MSUD-specific small RNA generation (64), although its participation in the complex has not been proven.

That quelling and MSUD both employ specific and common RNAi components, such as DCL-1 and QIP, suggests that quelling and MSUD may have evolved from an ancestral RNAi mechanism that has specialized throughout evolution (67). However, MSUD occurs only in ascomycetes because proteins homologous to some members of the MSUD pathway are absent in *Basidiomycota* and *Mucoromycotina* fungi (68, 69). On the other hand, additional RNAi components in both fungal groups are without a recognized function, suggesting that some other pathways, currently unknown, could function as genome defense during the sexual cycle. In fact, silencing by unpaired DNA during meiosis has been observed in numerous species of animals, including *C. elegans*, *Drosophila melanogaster*, and mice, indicating that it is a highly conserved phenomenon (70, 71).

For a long time, it was suggested that MSUD protects the genome by controlling the spread of mobile elements present in only one parent, because they will be unpaired during sexual crosses and thus become natural substrates for meiotic silencing (72). This hypothesis received strong support when the active DNA-type transposon Sly1-1 was identified in the genome of the most widely used laboratory *N. crassa* wild type strain. Deep sequencing of small RNAs accumulated during meiotic development detected masiRNAs that originated from Sly1-1. Furthermore, the meiotic silencing machinery is required for the production of masiRNAs emanating from Sly1-1 (73), suggesting that its transposition during meiosis is prevented by MSUD.

Heterochromatin formation

Heterochromatin is a condensed form of chromatin that is of fundamental importance to the regulation and stability of eukaryotic genomes. Constitutive heterochromatin covers regions of the genome containing highly repetitive sequences, mainly derived from mobile elements, that prevent transcription as well as recombination. In this context, the fission yeast *S. pombe* offers unique advantages for investigating the basic mechanisms that underlie heterochromatin formation because there are conserved pathways of heterochromatinization between fission yeast and multicellular eukaryotes (74). *S. pombe* chromosomes contain extensive heterochromatic regions that are associated with underlying repetitive DNA elements at the centromeres, subtelomeric regions, and silent mating-type loci (75). Formation of heterochromatin in these regions requires RNAi components, because mutations in the corresponding genes result in a loss of centromeric heterochromatin (76). The nucleation process is triggered by transcription during S-phase by Pol II of the repeats named dg and dh, which are characteristic of the heterochromatin domains (76). The noncoding transcripts produced at

these genomic regions are then reverse-transcribed into dsRNAs by the RNA-directed RNA polymerase complex, which includes the single RdRP of *S. pombe* (77). The dsRNAs are processed by the single Dicer enzyme into siRNAs that are loaded onto the RNA-induced transcriptional silencing complex (RITS), which includes the single AGO protein of *S. pombe*. RITS is targeted to the repeat regions through base pairing between siRNAs and the nascent transcripts, reinforcing silencing by facilitating the localization of the RNA-directed RNA polymerase complex (78). Moreover, RITS recruits the Clr4 methyltransferase complex that provokes the H3K9 methylation of this region, which is required to recruit the HP1 family proteins Swi6 and Chp2 and subsequently histone deacetylases such as SHREC to further compact chromatin (79).

One of the unknown parts of this process is how the noncoding transcripts produced by the heterochromatin domains are recognized by the RNAi components to trigger the whole process. One hypothesis suggests that dsRNAs are initially produced by bidirectional transcription of the centromeric repeats (76). Alternatively, the components of the spliceosome could provide a platform for RNAi because several proteins involved in splicing are required for centromeric heterochromatin formation (80, 81). Some of these proteins were found to associate with pericentromeric chromatin and with the RNAi element Cid12, one of the components of the RNA-directed RNA polymerase complex (80). The connection between RNAi and mRNA processing has been also reported in *C. neoformans*. In this fungus, transcripts with suboptimal introns show abnormally high occupancy of spliceosomes that, in this case, trigger RNAi-dependent degradation of the transcripts via a complex named SCANR (spliceosome-coupled and nuclear RNAi), which contains the single RNAi components of *C. neoformans* (RdRP, AGO, and QIP) and the splicing component Srr1 (82). Interestingly, noncoding RNAs produced from centromeric repeats undergo inefficient splicing that could result in either accumulation of the splicing machinery itself or possibly splicing intermediates, which may serve as a signal to recruit the RNAi machinery to target transcripts. A phenomenon reminiscent of this is present in the RNAi-less yeast *S. cerevisiae*, in which inefficient splicing seems to act as a regulatory signal tagging transcript for degradation by the RNA surveillance machinery (83). Connections between RNAi and splicing factors have been found in plants (84), worms (85), and flies (86), suggesting that recruitment of RNAi components by splicing machinery could be widespread in eukaryotes.

Regulatory Functions of RNAi

Although RNAi is best known for its protection of the genome against foreign nucleic acids, particularly transposons, it is also involved in the regulation of fungal physiology and development. This latter function of RNAi in fungi is less well characterized due to the absence of obvious physiological phenotypes in the RNAi-defective mutants in most fungal species. In this context, the *Mucoromycotina M. circinelloides* has recently become a valuable model because mutants in RNAi components show clear phenotypes that affect its physiology and development. Moreover, it uses several regulatory pathways (Fig. 1), which will be described in the next sections, to regulate the expression of a large number of genes (11).

Endogenous small RNAs involved in gene regulation

RNAi-mediated regulation is associated with noncoding endogenous small RNAs (esRNAs) of about 20 to 30 nt. The most relevant example of regulatory esRNAs is the animal and plant miRNAs, a class of esRNAs that are produced from hairpin-structured RNAs and block the translation or provoke degradation of target mRNAs (87). For a long time, miRNAs have been considered to be absent in fungi, but the application of deep sequencing technologies has revealed the existence of esRNAs similar to miRNA, named miRNA-like small RNAs (milRNAs) (88), in several ascomycetes and basidiomycetes (45). However, so far, none have been clearly linked to a particular physiological or developmental process. In addition to milRNA, several additional types of esRNAs have been identified, some of them with clear regulatory functions such as the *M. circinelloides* ex-siRNAs (see below).

milRNAs

The milRNAs were initially found and exquisitely characterized in *N. crassa*, which has at least four classes of milRNAs according to their mechanisms of biogenesis that use different combinations of RNAi components, including the two Dicer enzymes, QDE-2, QIP, and the putative RNase III domain-containing protein MRPL3 (88). Despite how they are produced, these milRNAs are similar to animal and plant miRNAs because they are predominantly derived from one strand of hairpin-like single-stranded RNA precursors, with a strong preference for U at their *5′* termini. Most interestingly, *N. crassa* milRNAs can repress the expression of endogenous genes, and levels of the predicted miRNA target genes are upregulated in RNAi mutants, suggesting that

milRNAs regulate gene expression. Thereafter, milRNAs have been identified in an increasing number of dikaryotic species, i.e., *Ascomycetes* and *Basidiomycetes*, but not in basal fungi, including *Mucoromycotina* species (45). Despite the presence of milRNAs in many fungi, their physiological importance remains to be determined.

Nat-siRNAs and disiRNAs

Natural antisense transcripts (NATs) are noncoding RNAs complementary to sense RNAs. The presence of NATs in eukaryotic genomes is widespread, and studies of several eukaryotes have demonstrated that they participate in the regulation of their complementary sense transcripts via a range of mechanisms, including RNAi (89). They are also present in fungi, but the connection with RNAi is not clear, except for yeasts, in which overlapping sense and antisense transcripts form dsRNAs that are recognized by the RNAi machinery and processed into siRNAs (90, 91). These siRNAs are named nat-siRNAs, from NAT-derived siRNAs, to distinguish them from the siRNAs produced from RdRP-derived dsRNAs. However, *N. crassa* accumulates Dicer-independent small interfering RNAs (disiRNAs) that are also derived from overlapping transcripts. Despite having canonical siRNA characteristics (22 nt long and a 5′ uridine bias) and being bound by AGO protein QDE-2, their biogenesis is independent of all canonical RNAi components (Dicers, AGO, and RdRP), suggesting that they are produced by an unknown RNAi-independent mechanism (88). Interestingly, disiRNAs arise from several gene-rich regions, where they mediate a new type of transcription-dependent DNA methylation event (disiRNA loci DNA methylation), which is triggered by convergent transcription and enriched in the promoter region (92). Moreover, disiRNAs also mediate the recruitment of enzymes that modify heterochromatin to establish facultative heterochromatin regions at promoters to regulate the expression of sense target transcripts. This type of regulation has been observed in the circadian gene *frq* of *N. crassa*, and it is needed to establish the appropriate circadian phase. However, the molecular mechanism of disiRNA-mediated heterochromatin formation is still unknown (93).

esRNAs of *M. circinelloides*

The *Mucoromycotina* fungus *M. circinelloides* represents an outstanding fungal model because of its evolutionary distance from classical dikaryotic models, providing a different perspective on fungal biology. This fungus accumulates esRNAs enriched in exonic sequences compared with intergenic and repetitive regions, which is in contrast to other fungi, in which most esRNAs are produced from repeats and transposons (12). A large number of these exon-derived esRNAs, named ex-siRNAs, are generated by Dicer-dependent RNAi pathways (Fig. 1). These ex-siRNAs are heterogeneous in structural characteristics and biogenesis pathways. Thus, four classes of ex-siRNAs (classes 1 to 4) have been defined according to the combination of the silencing machinery components required for their biogenesis (9, 12), which define four RNAi pathways (94). Classes 1 and 2 are produced by canonical RNAi pathways and show characteristics of functional siRNAs such as a defined size of 23 to 24 nt with no strong strand bias, binding to the Ago-1 protein, and a very strong bias for uracil at the 5′ position of the ex-siRNAs. Unlike classes 1 and 2, class 3 and 4 ex-siRNAs are not bound by Ago-1 and show different structural features that suggest they are produced by a noncanonical RNAi pathway (12). The crucial breakthrough in the *M. circinelloides* RNAi field was the discovery that ex-siRNAs regulate the expression of the protein-coding genes from which they are derived by guiding degradation of the corresponding mRNAs (74). The existence of both a large number of genes regulated by ex-siRNAs (11) and clear-cut phenotypes of mutants for strains lacking RNAi components indicate that ex-siRNAs are regulatory esRNAs generated by Dicer-dependent RNAi pathways. Exon-derived esRNAs with putative regulatory functions have also been described in the ascomycete *Trichoderma atroviride*. Similar to *M. circinelloides*, differential accumulation of some exon-derived esRNAs correlates with differential accumulation of the corresponding transcripts (95).

In addition to Dicer-dependent ex-siRNAs, *M. circinelloides* accumulates a significant amount of esRNA generated by a noncanonical *dicer*-independent but *rdrp-1*- and/or *rdrp-2*-dependent RNAi pathway, in which an RNase III-like protein called R3B2 plays a crucial role (Fig. 1). Most of the esRNAs produced by the *dicer*-independent *rdrp*-dependent RNAi pathway correspond to exons and show characteristics that suggest that they are nonrandom degradation products of specific mRNAs. Therefore, they were named *rdrp*-dependent degraded RNA (rdRNA). Interestingly, this new noncanonical RNAi pathway regulates mRNA levels, because a reduction in rdRNA levels correlates with an increase in mRNA accumulation of the corresponding genes (96). Moreover, R3B2 is an interesting protein because it sports an unusual domain, formed by one RNase III and two dsRNA-binding domains, which

defines an RNase family specific to the *Mucorales*. In addition to being required for the production of esRNAs by the noncanonical RNAi pathway, it is also essential for the biogenesis of several classes of *dicer*-dependent ex-siRNAs (96), suggesting that it might also interact with Dcl-2 to positively affect Dicer activity in ex-siRNA generation (Fig. 1) (94).

Physiological and developmental responses governed by *dicer*-dependent RNAi pathways

Mutants in each of the core components of the *M. circinelloides* RNAi pathway exhibit defects in physiology or development, which are probably a consequence of altered expression of genes regulated by esRNAs (11). Several phenotypes are shared for more than one mutant, suggesting that the esRNAs generated by particular combinations of RNAi proteins orchestrate particular physiological and developmental responses. This section deals with the processes controlled by different groups of *M. circinelloides dicer*-dependent ex-siRNAs that include vegetative growth and hyphal morphology (10), asexual sporulation (9, 11, 28), and hyphal autolysis in response to nutrient starvation (9). In a similar way, *T. atroviride dcr2* and *rdr3* genes drive reproductive conidiation, while *dcr1* together with *dcr2* promotes vegetative growth, suggesting that these processes are also regulated by *dicer*-dependent exonic esRNAs in this fungus (95). In both fungi, these cellular processes are impacted by environmental signals such as nutrient availability, suggesting that RNAi pathways may mediate responses to environmental signals in other fungi (45, 97).

Analyses based on deep sequencing technology have identified several hundred genes in *M. circinelloides* that are probable targets of the *dicer*-dependent ex-siRNAs. Interestingly, the patterns of mRNA accumulation in different RNAi mutants fit quite well with the patterns of ex-siRNAs, suggesting that each class of ex-siRNA regulates the expression of a particular group of genes that control a specific physiological or developmental process. This is clearly observed in asexual sporulation and hyphal autolysis. Both processes are affected in *dcl-2*, *ago-1*, and *rdrp-2* mutants, which present altered expression of a common group of genes coding for proteins that are involved in signal transduction or regulation and in specific metabolic pathways associated with asexual sporulation and/or autolysis in other fungi (11, 94). The *dcl-2*, *ago-1*, and *rdrp-2* genes are required for the biogenesis of class 1 ex-siRNAs (12), suggesting that this ex-siRNA class controls asexual sporulation and autolysis in response to nutrient starvation (11).

As expected for direct regulation, the mRNA levels of several target genes of *M. circinelloides* ex-siRNAs increased in RNAi mutants unable to produce the ex-siRNAs, although a higher number of genes showed a decrease in transcript levels, suggesting they are secondary targets (11, 45). The existence of secondary targets, which has also been detected in *T. atroviride* (95), implies an amplification of the regulation by ex-siRNAs.

A dicer-dependent RNAi pathway is also involved in the virulence of *Botrytis cinerea*, although only part of the RNAi pathway implicated in pathogenesis operates in this fungus. In this process, this fungal pathogen transfers virulent sRNA into host plant cells to silence host genes in order to debilitate plant immunity and promote infection. These mobile sRNAs are processed by the two partially redundant Dicer proteins of *B. cinerea* from long RNA precursors derived from clusters within long-terminal repeat retrotransposons in the fungal genome. The *B. cinerea* sRNAs transferred to plant cells structurally mimic plant sRNAs and hijack the plant RNAi machinery by binding to AGO proteins. After that, *B. cinerea* sRNAs target genes with complementary sequences that are involved in plant immunity against *B. cinerea* (98). In recent years an increasing number of examples of RNA-signal exchange have been described to occur between organisms of different kingdoms (99), suggesting that *B. cinerea* may represent only the tip of the iceberg and that other fungi could transfer or receive sRNAs from other organisms, including other fungi. Almost all aspects of this trans-kingdom silencing phenomenon, from the selection of specific sRNAs to be transported to the hijacking of the host RNAi machinery to silence target genes, remain poorly understood. However, transport of sRNAs by extracellular vesicles has been proposed as a candidate mechanism by comparison with the transport of sRNAs within the cells of an organism and given the accumulation of vesicular material in the interphase between the plant and pathogen (99).

Physiological and developmental responses operated by *dicer*-independent RNAi pathways

Two cellular processes, response to oxidative stress and sexual interaction, are regulated by the *rdrp*-dependent *dicer*-independent RNA degradation pathway in *M. circinelloides*. Mutants in genes of this pathway, i.e., *rdrp-1*, *rdrp-2*, and *r3b2*, show increased resistance to oxidative stress and reduced production of zygospores, the sexual structures formed when mycelia of opposite mating types interact (96). The existence of complex physiological and developmental processes

altered in mutants of this degradation pathway, reinforced by the nonrandom generation of *rdrp*-dependent degraded RNAs, suggests that the *rdrp*-dependent *dicer*-independent RNAi pathway actively governs the expression of a specific set of genes, rather than just being an accessory RNA degradation mechanism. Many of the genes controlled by this pathway code for metabolic enzymes or proteins involved in regular cellular functions such as mRNA processing, translation, or signaling, including proteins associated with responses to different environmental stresses (28). Nevertheless, the high number of genes targeted by this noncanonical pathway predicts other cellular processes regulated by this RNAi mechanism that likely are not directly visible under standard growth conditions (94).

Phenotypic plasticity mediated by epigenetic mechanisms

The reversibility of the RNAi mechanism may provide remarkable phenotypic plasticity for rapid adaptation to changing environments, and RNAi-mediated silencing of specific gene expression under adverse environmental conditions can offer an important selective advantage, because descendants of silenced strains can inherit and maintain this specific epigenetic gene repression as long as the adverse environmental conditions continue to be present. If the environmental conditions change, the RNAi mechanism can interrupt repression of target genes, and the silenced strains can reverse to the original phenotype. This adaptive mechanism has been observed in *M. circinelloides*, in which the canonical RNAi pathway silences one specific gene to generate epimutants that are drug resistant (100). *M. circinelloides* develops spontaneous resistance to the antifungal drug FK506 via two distinct mechanisms. One involves Mendelian mutations that confer stable drug resistance, whereas the other is activated via an epigenetic RNAi-mediated pathway that results in unstable drug resistance. FK506 blocks the transition to hyphae and enforces yeast growth by interaction with the peptidyl-prolyl isomerase FKBP12 to form a complex that inhibits the protein phosphatase calcineurin (101, 102). Mutations in either the *M. circinelloides fkbA* gene encoding FKBP12 or the calcineurin *cnbR* or *cnaA* genes confer FK506 resistance, restoring hyphal growth in the presence of this drug. In parallel, RNAi is spontaneously triggered to silence the *fkbA* gene, producing drug-resistant epimutants. FK506-resistant epimutants are readily reverted to the drug-sensitive phenotype when selection is released by growth without this antifungal drug (100). Retention of the RNAi machinery in most fungi leads to the possibility that this epigenetic mechanism of rapid adaptation may operate in other fungi.

LOSS OF RNAi

The functional diversity of RNAi pathways supports the idea of positive selection and evolutionary advantages for the organisms that have retained this mechanism. Surprisingly, several organisms, including some fungi, lack an active RNAi mechanism due to the loss of *dicer*, *argonaute*, or both genes. The loss of RNAi in fungi affects the subphyla *Saccharomycotina* and *Ustilaginomycotina*, class *Wallemiomycetes*, and some fungi of the phylum *Microsporidia* (13), in addition to some species such as the highly virulent *Cryptococcus deuterogattii* (44, 60). The absence of a functional RNAi pathway raises the question about the advantages of its loss. The answer to this question might rely on the context in which these organisms had to evolve. For instance, there are yeasts such as *S. cerevisiae* and other filamentous fungi that are susceptible to be infected with "killer virus," a dsRNA virus that can be cytoplasmically inherited. This virus produces a toxin that kills other noninfected cells, whereas it confers immunity to the cells making the toxin. However, the dsRNA genome of this virus is not compatible with an active RNAi mechanism, which turns out to be a disadvantage under these circumstances. This could explain the existence of several RNAi-defective species in which loss of this virus defense mechanism confers a selective advantage over its RNAi-competent neighbors (103). Also related to viral infections, some microbial pathogens retain the genome of infective viruses because they confer new virulence mechanisms, improving their pathogenicity. This is the case for some trypanosomatid protozoans such as *Leishmania* species, which present increased virulence when they are bearing dsRNA viruses named LRV (*Leishmania* RNA virus) (104, 105), but this phenomenon has not been observed in fungi.

A third situation in which loss of RNAi becomes a selective advantage might be the case of parasitic organisms that are continuously evolving to escape from host defenses. These organisms need a high genome plasticity that ensures a high genetic diversity in the population. One way of ensuring this genome plasticity is allowing the movement of retrotransposable elements by loss of an active RNAi mechanism. This mechanism of rapid evolution based on loss of RNAi has been proposed in some strains of *C. deuterogattii* with increased virulence (60). Comparison of RNAi-deficient strains of *C. deuterogattii* with RNAi-proficient genomes of

the *Cryptococcus* pathogenic species complex revealed that a set of conserved genes, several of them playing roles in RNAi pathways, were lost in all *C. deuterogattii* isolates examined, suggesting that RNAi loss could have contributed to its pathogenic trajectory (44). In addition to the movement of retrotransposon elements, loss of RNAi also can benefit genome plasticity by extrachromosomal gene amplifications, which has been described in RNAi-deficient *Leishmania* (104). In summary, RNAi loss might seem an ingenious adaptation solution for the species described here. However, the deleterious effects of active transposons and the lack of an antiviral defense mechanism might imply a risk of extinction in the future of these species.

A HYPOTHESIS ON THE ORIGIN OF RNAi

RNAi has been a hot topic in biology since its discovery, becoming a milestone in the progress of understanding the complex biological mechanisms underlying gene regulation. Despite all of the accumulated knowledge, an explanation of the origin of this complex mechanism still remains elusive. The answer to this question might rely on the possible link between RNAi and the mechanism of standard mRNA decay, a more ancestral mechanism that shows evolutionary conservation across all domains of life. The first link between these two mechanisms showed how they compete for aRNA. Mutants with an impaired mRNA decay mechanism show boosted efficiency of transgene-induced gene silencing, indicating that degradation of aRNAs limits their entry into the RNAi pathway (106–108). Another link forged between these otherwise distinct RNA decay mechanisms is the discovery of a noncanonical *rdrp*-dependent *dicer*-independent RNAi pathway that is involved in degradation of specific mRNAs in *M. circinelloides* (Fig. 1) (96). This pathway could represent an intermediate evolutionary state between mRNA decay and RNAi, because a typical RNAi component, the RdRPs, participate in RNA degradation. The way RdRPs discriminate between canonical RNAi and RNA degradation pathways could be related to the transcriptional level of the target RNA. Thus, a high transcriptional level results in overproduction of aRNA, and an excess of aRNA could drive RdRP to activate the noncanonical degradation pathway (109–111). Accordingly, most genes regulated by the *rdrp*-dependent *dicer*-independent degradation pathway are highly expressed (96). In addition, when the noncanonical degradation pathway is blocked in *rdrp-1* mutants, the excess of aRNAs enhances the efficiency of the canonical RNAi pathway, and similarly it was enhanced by mutations in the canonical mRNA decay pathway (100). In summary, the dual role of RdRPs in the mechanisms of RNA degradation and canonical RNAi of *M. circinelloides* could be the initial step in the evolution of the RNAi system, followed by the appearance of Dicer enzymes with the role of processing the dsRNA generated by RdRPs. Finally, AGO proteins would have evolved to convert the siRNAs generated by Dicer into the effector molecules of the RNAi mechanism. Functional diversification would come later in multicellular eukaryotes, generating the complexity of the modern RNAi world (96).

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Amyloid Prions in Fungi

32

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INTRODUCTION

In 1994, a paper signed by a single author based on genetic approaches opened a decisive breach leading to a dramatic expansion of our perception of the biological significance of the prion phenomenon (1). In the following years, biochemical reconstitution and transformation established that these biological entities, originally identified and defined in the context of mammalian diseases such as Kuru or Creutzfeldt-Jacob disease, also exist in yeast as "protein-based genes" and correspond to previously described non-Mendelian genetic elements (2, 3). It is now clear that in most known cases the physical basis for prion propagation is the formation, growth, and fragmentation of an amyloid aggregate. Amyloids are ordered protein polymers with a so-called cross-β structure (4). The original definition of prions as "infectious proteinaceous particles" is imprecise enough to still be operational today but as a consequence embraces a variety of biological phenomena and structural features (5). Defining prions thus remains a nontrivial task. While a more restrictive definition is perhaps neither possible nor desirable, this general term induces some confusion and controversy. In an attempt to clarify discourse, at some point investigators in the mammalian disease-related field denied the fungal "infectious proteinaceous particles" the name of prions and proposed instead to term them "prionoids" (6). These semantic battles should not be disregarded as sterile, but rather should be taken as an indication of the variety of the biological realities that the term covers.

Prions are not necessarily harmful entities, nor do they necessarily correspond to amyloid structures (7). By some definitions they do not need to correspond to propagation of a specific conformational state (8). The situation is further complicated by the description of so-called prion domains; prion-forming domains (PFDs) are protein domains that confer the ability to other proteins to behave as prions in an *ad hoc* experimental setting. However, this observation does not necessarily imply that the native protein from which the domain is isolated also behaves as a prion (9, 10). That said, this chapter provides a description of prions and prion domains in the fungal kingdom. Our minimal operational definition of a prion here will be a protein able to adopt a transmissible conformation, this conformation being transmitted vertically to mitotic (and often meiotic) progeny and horizontally between strains through cytoduction (in yeast) or somatic anastomosis (in filamentous fungi) (Fig. 1). We will more specifically focus on models for which the amyloid nature of the prion particle is known or can be suspected.

The study of prions in fungi is largely that of prions in the yeast *Saccharomyces cerevisiae*, but a prion and several PFDs have also been identified in filamentous fungi. Three historical prion models, [URE3], [*PSI*$^+$] in yeast, and [Het-s] in *Podospora anserina*, were identified and studied as non-Mendelian genetic elements before they were found to correspond to prions (11–13). [*PIN*$^+$], in turn, was identified in relation to prion curing experiments carried out on [*PSI*$^+$] strains (14). The list of fungal prions now amounts to about a dozen (15). The work on yeast prions dominates the literature in terms of sheer amount, diversity of models, and level of detail. In many aspects, yeast models and the [*PSI*$^+$] system have established the general paradigms and methods that are used in the field. Prions from filamentous fungi (that is, essentially the [Het-s] model), are considered by comparison to this paradigm, and in some aspects, but not all, they conform to it. Yeast prion systems are also intensively used to study the aggregation and toxicity and prion behavior of proteins

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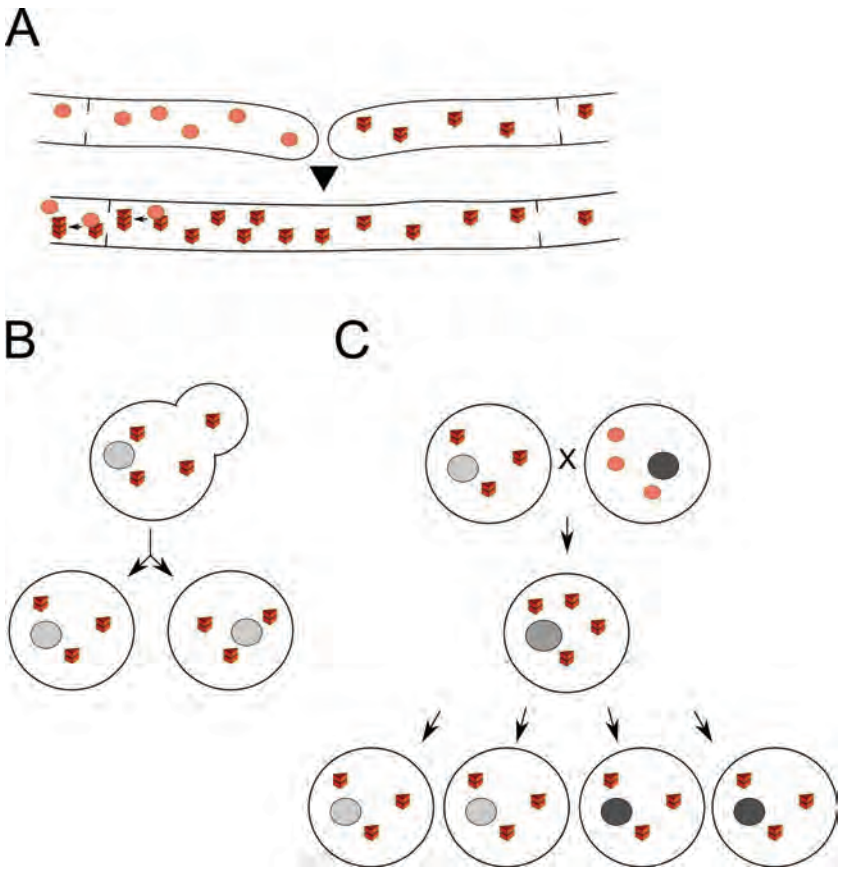


Figure 1 Natural situations of prion propagation in fungi. (**A**) Prion propagation after hyphal anastomosis in a filamentous fungus (for instance, [Het-s] in *Podospora anserina*). The prion form is transmitted from a donor-infected strain (right) to a recipient strain (left). The prion form then converts the entire mycelium to the prion state due to cytoplasmic continuity throughout the thallus. Prion transmission also occurs in meiotic crosses with maternal inheritance (not depicted here). (**B**) Prion propagation during mitotic cell divisions in yeast. Prion seeds are transmitted from mother to daughter cells during budding. (**C**) Prion transmission during sexual crosses. In a cross between a [PRION$^+$] (left) and a [*prion*$^-$] strain (right), the resulting diploid is [PRION$^+$] and there is non-Mendelian segregation of the [PRION$^+$] character (often, but not always, with 4:0 segregation as in the example depicted here).

or protein domains derived from other species. This approach has been used commonly, but not exclusively, for proteins relevant to human protein-deposition diseases (see references 16 and 17 for recent examples of this approach). This line of research will not be specifically discussed here.

PHYSIOLOGY OF PRION FORMATION AND PROPAGATION IN FUNGI

The initial discovery of prions in fungi, and immediate follow-up work, focused on cells being in one of two states: [*PRION*$^+$] and [*prion*$^-$]. Because their inheritance involves self-templating changes in protein conformation, [*PRION*$^+$]-based traits have very different patterns of inheritance in genetic crosses than DNA-based traits: their phenotypes are dominant (denoted by capital letters) and segregate to meiotic progeny in a non-Mendelian fashion (denoted by brackets). The characterization of [*PRION*$^+$] and [*prion*$^-$] states quickly moved beyond phenotypic assessment and epigenetic inheritance of the phenotype to focus on the aggregation of the prion protein itself. The aggregated state of the prion protein was followed by microscopy with fusion proteins and by sedimentation analyses that could discern soluble and insoluble, or higher molecular weight

species (2, 18–20). Below we highlight specific examples that laid the foundation for this burgeoning field.

[*PSI*$^+$]: The Most Studied Fungal Prion

The prion form of the translation termination factor Sup35 (eRF3) results in inefficient termination and detectable nonsense suppression (21). Strains harboring a premature termination codon in either the *ADE1* or *ADE2* genes (the *ade1-14* or *ade2-1* alleles) are unable to synthesize adenine. The loss of either Ade1 or Ade2 proteins in an otherwise functional pathway results in the buildup of a metabolic intermediate that gives the colonies a red color. Read-through of the premature termination codon (nonsense suppression) and completion of the pathway restores the colony color to white when nonsense suppression is high or to pink when it is not as high. Thus, *ade1-14* or *ade2-1* colonies are red when cells are [*psi*$^-$] and Sup35 is soluble and translation termination is efficient. They are white when cells are [*PSI*$^+$] and Sup35 is in the prion state.

Prion Variants and Prion-Forming Domains

The sensitive phenotype associated with the [*PSI*$^+$] prion enabled the discovery of distinct [*PSI*$^+$] prion variants (22). Different heritable states of [*PSI*$^+$] were observed without any genetic change. That is, they arose from the same Sup35 protein sequence. These states were termed prion "variants" and are analogous to mammalian prion strains. These are thought to be different self-propagating structures of the mammalian prion protein PrP that are largely responsible for variation in pathologies in mammalian prion diseases (23). [*PSI*$^+$] variants are discernable phenotypically by stable and distinct colony colors that range between red and white. How white or pink the colony is correlates to how much Sup35 is in the nonfunctional prion state. This can also be assessed biochemically by the relative amount of insoluble Sup35 protein in [*PSI*$^+$] variant lysates (21, 24–26).

Prion variants are distinct self-propagating structures of a prion protein. They are derived from the same polypeptide sequence. Thus, the stable phenotypic differences are a consequence of these structural changes. Proof of that concept was provided by the generation of biophysically distinct amyloid structures *in vitro* with purified recombinant Sup35 protein, which was then used to infect [*psi*$^-$] yeast and generate phenotypically discernable [*PSI*$^+$] variants (27). Such prion variants are not unique to [*PSI*$^+$] and have been observed with [URE3] (28, 29) and [*PIN*$^+$/*RNQ*$^+$] (30–32) as well. In addition, PrP has been suggested to replicate as over 30 prion strains. Many (perhaps all) amyloidogenic proteins associated with human disease can form multiple self-replicating β-sheet-rich structures (33). In contrast to what has been described for several yeast prion models, no prion variants have been reported for the [Het-s] system. One may argue that the lack of a sensitive detection assay has prevented identification of such hypothetical variants. Yet the fact that HET-s prion fibrils are characterized by the absence of structural polymorphism further supports the notion that there are no [Het-s] variants (34).

Distinct amyloid structures formed by the same protein involve different primary structure elements. Thus, changes in protein sequence can have differential effects on the propagation of distinct prion variants. For example, mutation of an amino acid residue that is buried in the core (packed in the templating β-sheet structure) of one amyloid structure but on the outside of the core in another amyloid structure can prevent propagation of one structure but have no effect on another (21, 35–39).

Although some mutants can prevent propagation of a particular amyloid structure, the ability to establish a prion state appears to be more dependent on the overall amino acid composition of the PFD (40, 41). For most of the prion proteins identified (particularly those in *Saccharomyces*), the PFD is a long stretch of amino acids rich in glutamine and asparagine residues. These domains are typically devoid of predictable secondary structure and are thought to be intrinsically disordered. Proteins with intrinsically disordered domains are not uncommon and are generally thought to acquire a stable structure upon interaction with a cofactor or complex (42). Such domains may render the protein susceptible to aggregation, however. Indeed, a wide spectrum of human protein conformational disorders may be caused by the aggregation of proteins with intrinsically disordered domains. Many of these harbor glutamine- and asparagine-rich sequences. Initial structure/function work to investigate regions of Sup35 required for prion formation determined that the amino terminal PFD was not only necessary, but also sufficient for prion propagation (43). The prion-conferring activity was transferable to a different protein by simply fusing the PFD to it (43). This appeared to be true for other PFDs as well, such as the Ure2 (44) and Rnq1 PFDs (45).

Chaperones Modulate Prion Formation and Propagation

Yeast allowed the identification of Hsp104 as the first chaperone to alter prion propagation (46). In fact, the activity of Hsp104 is required for most prions to

faithfully propagate and transmit from mother to daughter cells. Hsp104 works in conjunction with Hsp70 and Hsp40 cochaperones, primarily Ssa1/2 and Sis1. Hsp70 and Hsp40 work together to recognize misfolded or aggregated polypeptides and deliver them to Hsp104, which then unfolds substrates by ATP-dependent threading through a central pore in its hexameric structure (47–50). This system is required for the propagation of amyloid-based prions and is thought to generate smaller oligomeric amyloid seeds that not only increase the free ends for efficient propagation, but also more readily transit the mother-bud neck to maintain stable inheritance. The prion variant-specific chaperone activity requirements may stem from the ability of chaperones to recognize specific accessible sequence elements as well as the amount of energy needed to break up the amyloid structure to generate oligomeric seeds. While many of these effects are challenges that alter cellular chaperone balance in a specific, and possibly nonphysiologic, manner, one variant of the [*PSI*$^+$] prion can be cured by transient heat shock (51). In this case, prion propagation is altered by a global cellular reaction to stress that would mimic a naturally occurring chaperone response.

Prion-specific effects of chaperones have been reported as well. While Hsp104 has perhaps the most general effect on different yeast prions (though it does not affect all prions), its relationship to [*PSI*$^+$] is unique in that the overexpression of Hsp104 can cure cells of [*PSI*$^+$]. Most prions affected by Hsp104 are cured by its inhibition or deletion and are not affected by its overexpression. One plausible explanation for this relates to the necessity for activity of Sup35 for viability. *SUP35* cannot be deleted because some Sup35 activity is required for translation termination. This likely limits the array of [*PSI*$^+$] prion variants that can propagate and maintain cell growth. If Sup35 were to form a very efficiently propagating prion structure, cell viability would be compromised. Indeed, some data support this (52, 53). It is possible that the structural array of [*PSI*$^+$] variants and the requirement for a pool of soluble, functional Sup35 results in less flexibility in the amount of Hsp104 activity that can support [*PSI*$^+$] maintenance. There is some specificity in the Hsp70 involvement with Hsp104 in prion maintenance as well. Ssa1 is the Hsp70 required for the propagation of [*PSI*$^+$], while the nearly identical Ssa2 is required for the propagation of [*URE3*]. In addition, the Hsp70 nucleotide exchange factor Sse1 is required for the propagation of [*URE3*] but for only some variants of [*PSI*$^+$] (54, 55). Furthermore, some chaperones selectively impact the propagation of the [*URE3*] prion. The Hsp90/Cpr7 chaperone system is required for the propagation of [*URE3*] (56). Finally, Hsp42 overexpression also cures [*URE3*] in a manner that is dependent on a putative protein transport system that also shows some variant-specific effects on [*URE3*] propagation (57).

Prion-variant-specific effects of the chaperone machinery have also been reported. For instance, it was reported that the PFD of the prion protein Rnq1 is sufficient to propagate some [*RNQ*$^+$] variant structures, but not all. This may be primarily because there is a chaperone binding site in the non-PFD domain of Rnq1, and the binding of that chaperone, the Hsp40 Sis1, is required for the propagation of the [*RNQ*$^+$] prion (39). The reduction in Sis1 binding or activity cures the cells of the [*RNQ*$^+$] prion (58). Sis1 appears to interact tightly with Rnq1, only in the [*RNQ*$^+$] prion state (59), but interacts with every [*RNQ*$^+$] variant tested (31, 60). Moreover, Sis1 interacts with at least two regions of Rnq1 (39, 61). The function of Sis1 in [*RNQ*$^+$] propagation is tied to the activity of the Hsp104 disaggregase. Sis1 is also essential for the propagation of other prions tested, including [*PSI*$^+$] (62), and different variants of [*PSI*$^+$] show different requirements for Sis1 activity to propagate (62). The Hsp104 chaperone was also found to be involved in the propagation of the [Het-s] prion, where the propagation rate and the number of prion particles per cell is reduced in a mutant background for Hsp104 (63). The [Het-s] prion can also be propagated in yeast (64), indicating that the chaperone machinery is able to replicate a totally alien prion. In this setting also, Hsp104 was required for prion propagation.

There is also evidence for the involvement of other cellular machineries distinct from the chaperone network in the maintenance of prions. For instance, overexpression of the Btn2 protein involved in endosomal protein sorting cures [*URE3*] (57), and autophagy protects against [*PSI*$^+$] formation (65). In addition, the actin cytoskeleton and the ubiquitination systems have also been found to modulate prion propagation (reviewed in reference 66).

FUNCTIONS AND TOXICITY

Because the prion concept was initially conceived to explain a baffling pattern of disease transmission, the term has long been associated with pathology. However, in the intervening decades many examples of functional amyloids have been reported in a wide variety of organisms. Although their evolutionary value has been controversial (67, 68), multiple lines of evidence suggest that many fungal prions can exert both positive

and negative phenotypic effects, depending on strain and circumstance.

Functional versus Pathogenic Prions

Proteins with prion-like properties have been discovered in organisms ranging from yeast to mammals (15, 69). Their effects range from being necessary in non-self recognition ([Het-s] in *P. anserina*; see reference 70) to deadly (neurodegenerative diseases caused by PrP in humans, deer, and elk; see references 5 and 71). Computational analyses suggest that PFDs are ubiquitous in fungal proteomes (10, 72). Molecular characterization in *S. cerevisiae* indicates that some of these putative prions do indeed have the capacity to adopt multiple self-templating conformations under normal physiological conditions (10, 73). Their ubiquity, and the observation that fungal prions result in diverse phenotypes, some of which are adaptive (74–76), leads to a fundamental question about the biology of these epigenetic elements: Are prions purely selfish replicating elements, or can they provide some benefit to the organism? This question has been the subject of vigorous debate. Some argue that prions are often adaptive "bet-hedging" elements that can facilitate survival in fluctuating environments. Others have maintained that most fungal prions are simply diseases.

The bet-hedging hypothesis (68, 75, 77–79) is based on observations of phenotypic diversity imparted by prion switching and on the fact that rates of [*PRION*$^+$] acquisition and loss are much higher than the rate of spontaneous mutation. For example, within a [*prion*$^-$] yeast colony, a few [*PSI*$^+$] cells will appear sporadically, expressing heritable new phenotypes. If the [*PSI*$^+$] phenotype is detrimental, only a few individuals will be lost from a genetically identical population. If it is advantageous in a stressful environment, however, those few individuals might ensure survival of that genome when it would otherwise have been lost. [*PSI*$^+$] is also lost sporadically, providing a complementary survival advantage should the environment again change to favor the [*prion*$^-$] state. Thus, switching between the [*prion*$^-$] and the [*PRION*$^+$] states could enhance phenotypic diversity and promote survival of genetic lineages in fluctuating environments where they might otherwise have perished. Indeed, some have argued that repeated cycles of prion gain and loss (and indeed even spontaneous translation errors) may create a situation in which variation in the 3′-untranscribed regions (3′-UTR) of genes subject to [*PSI*$^+$]-mediated read-through have already experienced some selection (80).

Evidence supporting this hypothesis includes (i) the abundance of PFDs in fungal genomes (10, 72) and their common occurrence in regulatory proteins; (ii) prion induction and loss in stressful conditions when phenotypic variation would be most beneficial (81, 82); (iii) the existence of prion variants—akin to genic alleles (26, 30, 83–86); (iv) modular domain architecture (for example, Sup35's PFD does not appreciably affect translation termination but has been retained in fungi for ~500 million years [87]); (v) the recent appreciation that prion-based traits are common in wild yeast populations and that some of these are strongly beneficial (74); (vi) genetic assimilation of traits that are initially dependent on [*PSI*$^+$], which can be rendered independent of the prion via meiotic recombination of the cryptic genetic variation that initially drives them (74, 77)—this provides a means to separate the beneficial phenotype from the costs of the mechanism generating it; and finally (vii) because of their capacity to regulate translational fidelity throughout the transcriptome, the traits produced by [*PSI*$^+$] are genetically complex (75, 77) and would require far longer to achieve by mutation alone (see below).

Others have argued that most fungal prions are selfish elements, diseases, or even artifacts of laboratory cultivation (53, 67, 88) and that the rare beneficial phenotypes they induce are a side effect of infection. This line of thinking posits that the evolutionary retention of prion proteins can be explained because selection has been too weak to remove them (89). Evidence supporting this hypothesis includes (i) the fact that most individual prions are present at low frequencies in natural populations (74, 88); (ii) the occasional generation of lethal [*PSI*$^+$] variants *in vivo* when the prion and translation domains are separated (53); (iii) the observation that population allele frequencies of some prions are similar to those that would be expected for deleterious elements in outcrossing populations (89); (iv) the existence of structural variants for most prions, which could reflect an absence of positive selection because at least one prion, [Het-s], shows no such variation; (v) some PFDs having additional functions that could explain their evolutionary conservation (67); and (vi) prion acquisition leading to a stress response in some genetic backgrounds (90).

These hypotheses are not mutually exclusive, and most fungal prions show some degree of antagonistic pleiotropy. That is, they are beneficial in some environments and detrimental in others. Indeed, prions are clearly not universally beneficial, or they would have been fixed in natural populations of fungi. Standing frequencies of [*PSI*$^+$] and [*MOT3*$^+$] in natural populations of yeast (74) are consistent with a modest (~1%) fitness detriment on average for these elements (89).

These models necessarily make many assumptions about rates of [*PRION*$^+$] gain and loss and the frequency of outcross mating. Alternative values for these parameters, which have been observed in other studies (91), suggest a fitness benefit (69). Further investigation of these questions in the appropriate physiological context will be critical to resolve these questions. Computational modeling has also placed these arguments within a theoretical framework grounded in population genetics (79, 92, 93). These analyses suggest that [*PSI*$^+$]'s potential benefit as a bet-hedging element would not likely be sufficient to explain its evolutionary retention under nonstressed conditions. In contrast, bet-hedging would be sufficient to motivate retention of [*PSI*$^+$] in stressful environments, even with realistic rates of sex. The selective advantage calculated with these models is even stronger for another prion, [*GAR*$^+$] (see below).

Yeast Prions in (Epi)Genetic Diversity and Prions in Biotic Interactions

[*PSI*$^+$] is formed by the translation-termination factor Sup35, which ensures faithful termination by the ribosome at stop codons. The regions downstream of such stop codons (i.e., in the 3′-UTR of genes) are comparatively free to accumulate mutations because they are not under selective pressure to produce a protein (although they often are subject to selection as regulatory sequences). Acquisition of [*PSI*$^+$] causes translational read-through of many stop codons throughout the transcriptome (94) and consequently drives the emergence of new heritable phenotypes. This process is conceptually analogous to duplicated genes that are inactivated by a premature stop codon and are retained as pseudogenes, providing a source for the generation of new functional gene products via mutation and eventual reactivation (95).

This prion-dependent phenotypic diversification is strongly dependent on genetic background. That is, a phenotype elicited by [*PSI*$^+$] in one strain background might not be elicited in another. For example, in *S. cerevisiae* strain 33G the acquisition of [*PSI*$^+$] led to increased resistance to bleomycin and sensitivity to benomyl. In contrast, acquisition of [*PSI*$^+$] led to sensitivity to bleomycin and resistance to benomyl in *S. cerevisiae* strain 5G (75). Genetic dissection established that [*PSI*$^+$]-induced changes in translational fidelity produce new phenotypes based on genetic variation that was previously silent or "cryptic" (75, 77). Most phenotypes produced by [*PSI*$^+$] are driven by multiple polymorphisms. [*PSI*$^+$] thus provides immediate access to traits that otherwise require the serial acquisition of multiple rare mutations. The degree to which this mechanism has contributed to the evolution of biological novelty remains to be established. However, the presence of [*PSI*$^+$] in wild strains of *S. cerevisiae* (74) and its capacity to fuel strong phenotypes in those backgrounds suggest that this prion has exerted an important influence on the phenotypic landscape of natural fungal isolates.

In the 2 decades since [*PSI*$^+$] was recognized as a prion, approximately 10 other fungal prions have been discovered (15). Some, such as [*OCT*$^+$], formed by the Cyc8 transcriptional repressor (96), were found serendipitously. Others, such as [*MOT3*$^+$], formed by the Mot3 transcription factor, were found in a systematic screen to identify proteins with N/Q-rich sequences that resembled three other prion proteins known at the time: Sup35, Rnq1, and Ure2 (10). Investigation of [*MOT3*$^+$] revealed that it governs the acquisition of facultative multicellularity in *S. cerevisiae*, likely through both gains and losses of function (73). This phenotype is driven by [*MOT3*$^+$]-dependent transcription of *FLO11*, a major determinant of cell-cell adhesion, in response to nutrient deprivation. [*MOT3*$^+$] can be regulated by the environment. It is induced in response to ethanol and lost in response to hypoxia, providing some suggestion that it may be engaged in a subpopulation of cells during natural respiro-fermentative cycles (73). These phenotypes (in addition to others produced by the prion [see reference 74]) are highly strain-dependent. Thus, in addition to [*PSI*$^+$], [*MOT3*$^+$] can exert a strong influence on the phenotypic manifestation of natural genetic variation.

For decades, prions that create new traits (e.g., [*PSI*$^+$] and [*MOT*$^+$]) had not been found in wild strains of *S. cerevisiae* (88). Although these studies were limited in scope, they led to the perception that prions could be rare diseases or artifacts of laboratory cultivation. However, an examination of hundreds of wild *S. cerevisiae* isolates revealed that ~1 to 2% harbored [*PSI*$^+$] and ~6% harbored [*MOT3*$^+$] (74). These elements conferred many beneficial phenotypes under diverse selective conditions. Approximately one third of the wild strains examined in this study had heritable phenotypes that required the activity of Hsp104 to be propagated from one generation to the next. Those that were tested could also be transmitted from one strain to another through cytoplasmic mixing without transferring nuclear material. That is, these traits had prion-like patterns of propagation. Although the molecular origin of these traits remains to be identified, these experiments established that a potentially broad array of prion-like elements can influence the phenotypes of wild strains.

Switching between [*prion*$^-$] and [*PRION*$^+$] states is generally thought to be a rare event. For example, although the rates of [*MOT3*$^+$] acquisition are increased in response to ethanol, most cells still do not switch under these conditions (73). The [*GAR*$^+$] prion provides a striking exception to this paradigm. Discovered by chance decades ago in a screen for mutants that reverse glucose repression (97, 98), [*GAR*$^+$] is a protein-based element of inheritance that allows fungi (*S. cerevisiae* and other related yeast species including *Nauvozyma castellii* and *Dekkera bruxellensis*) to circumvent a normal hallmark of their biology: extreme metabolic specialization for glucose fermentation (7, 99, 100).When glucose is present, even in trace quantities, yeast will not metabolize other carbon sources. [*GAR*$^+$] allows cells to circumvent this "glucose repression," and arises at different frequencies in wild fungal isolates. These parameters strongly correlate with the ecological niche from which the strain was derived (100).

Through serendipitous contamination of a selective plate it was discovered that switching to [*GAR*$^+$] can be induced in virtually all *S. cerevisiae* cells when they are cocultured with certain species of bacteria (99). This cross-kingdom communication proceeds through an as yet unidentified small molecule secreted by the inducing bacteria. The results of this communication benefit yeast and bacteria alike. Providing an advantage for the bacteria, [*GAR*$^+$] yeast cells produce less ethanol than [*gar*$^-$] yeast cells. Providing an advantage for the yeast, [*GAR*$^+$] cells can readily metabolize complex carbohydrates and survive better in late-stage fermentations. As expected for a mechanism whose adaptive value originates from the selective pressures of life in biological communities, the ability of bacteria to induce [*GAR*$^+$] and the ability of yeast to respond to bacterial signals have been lost repeatedly during the monoculture inherent to laboratory domestication. These data suggest that [*GAR*$^+$] is a broadly conserved and often adaptive strategy to link environmental and social cues to heritable changes in metabolism.

Prion Domains in Signal Transduction in Filamentous Fungi

In the filamentous fungus *P. anserina*, the prion protein HET-s is involved in a cell death process known as heterokaryon incompatibility (70). Such incompatibility arises when a strain bearing the [Het-s] prion (in its amyloid form) and a strain expressing a different allelic variant of this protein (termed HET-S) come into contact. The [Het-s] prion is common in natural isolates of *P. anserina*, leading to the prevailing view that the prion is adaptive (66, 101). HET-s and HET-S are two-domain proteins with an N-terminal globular domain and a C-terminal prion-forming domain. Interaction with [Het-s] causes HET-S to relocalize from the cytoplasm to the cell periphery (102). Biochemical and structural studies suggest that this interaction drives conformational conversion of a HET-S PFD region into a β-solenoid fold which in turn induces a refolding of the globular HeLo domain (Het-s/LOP-B; see more on structure in the next section) to expose an ~34-residue transmembrane segment at the N-terminus of the protein. This converts HET-S into an integral membrane protein. Once it is at the membrane, HET-S oligomerizes and fuels the loss of membrane integrity in a manner resembling pore-forming toxins (103). The role of this type of conformational conversion in signaling intrinsic programmed cell death is only beginning to be appreciated. The *P. anserina* genome contains a gene encoding a Nod-like receptor protein (NWD2) adjacent to the gene encoding HET-S (104). Strikingly, NWD2 contains an N-terminal region that is homologous to the amyloid motif of HET-s. When NWD2 binds its cognate ligand, this interaction also drives conversion of HET-S into the amyloid conformation (105).

The paradigms established by the study of HET-s appear to be echoed in programmed cell death in other fungi. The short prion motif and globular HeLo domain characteristic of HET-S are also associated with other proteins such as lipases and regulatory Nod-like receptors in other fungi. The functional and evolutionary significance of this relationship has recently been examined in the saprophytic fungus *Chaetomium globosum* (106). A cluster of genes harboring short prion motifs was examined that included a protein known as HELLP (because it contains a HeLo-like domain). HELLP also has an N-terminal transmembrane helix that is homologous to that of HET-S. Despite the evolutionary distance between this organism and *P. anserina*, HELLP behaves as a HET-S analog, relocating to the membrane upon interaction with the prion form of the short prion motif. Moreover, the HeLo-like domain of HELLP bears homology to mammalian pore-forming domains involved in necroptosis, suggesting the possibility of an ancient evolutionary relationship between these processes.

Amyloid/Prion Toxicity in Yeast

The toxicity of prion amyloids in yeast has been the subject of intense debate. Most commonly studied variants of [*PSI*$^+$] have a slight fitness defect. However, overexpression of the NM domain of Sup35 induces [*PSI*$^+$] variants with a wide spectrum of effects. Those that are lethal are normally purged from the population

but can be maintained when the C-terminus of Sup35 (Sup35C, which cannot be converted into [*PSI*$^+$] but is competent for translation termination) is expressed on a plasmid (53). In these experiments ~8% of the induced [*PSI*$^+$] variants were lethal. The remaining variants were either slow-growing or nontoxic. The slow-growing variants were unstable upon loss of the Sup35C plasmid. In contrast, the nontoxic [*PSI*$^+$] variants were stable. Analogous results were seen with [URE3] in these experiments, highlighting the fact that selection has already operated on the ensemble of prion conformers typically studied in the laboratory. The molecular interactions governing prion loss remain to be fully elucidated but involve multiple chaperone activities including Hsp104, Hsp42 Btn2, and Cur1 (57).

In other cases, prion proteins are toxic only when overexpressed, and only in [*PRION*$^+$] cells. For example, Rnq1 is profoundly toxic in [*RNQ*$^+$] but not [*rnq*$^-$] cells (61). This toxicity is not caused by a general proteotoxic stress. Rather, in these cells Rnq1 sequesters the core spindle pole body component Spc42 in an insoluble protein deposit, engaging the Mad2 cell cycle checkpoint. The Hsp40 chaperone Sis1 suppresses this toxicity, but rather than inhibiting aggregation of Rnq1 it drives assembly of the protein. Interfering with this Sis1-triggered aggregation exacerbates Rnq1 toxicity. These data underscore the fact that amyloids are not always the toxic species in prion-like aggregation pathways. Molecular understanding of these distinctions, as well as the structural differences between toxic and nontoxic amyloid species stand as goalposts for future work.

FUNGAL PRION STRUCTURES

Structural characterization of amyloids, which constitute the physical basis of many fungal prions, is a considerable challenge, and one faced by the amyloid field in general. The most adapted technique to gain access to high-resolution structures of amyloid assemblies is currently solid-state nuclear magnetic resonance (NMR) (107). Other approaches include X-ray diffraction techniques that so far can only inform on the overall fold or fold modification. The Eisenberg group has also reported high-resolution X-ray crystallography structures of seven amino acid peptides derived from the Sup35 PFD assembled into nanocrystals (108), but this approach cannot be applied to the full-length PFD so far. One of the central limitations hindering progress in the structural determination of amyloids is structural heterogeneity. It appears that many amyloids, in particular disease-related amyloids but also yeast prion models, exist as mixtures of structural polymorphs displaying subtle or large differences in their amyloid architecture (109).

The [Het-s] prion is currently the only model for which a high-resolution structure is available. Solid-state NMR could be successfully applied here, because HET-s PFD fibrils are not polymorphic and lead to well-resolved solid-state NMR spectra. The HET-s PFD adopts a so-called β-solenoid structure with two rungs of β-strands per monomer. This structure resembles other β-helical structures found in soluble proteins (110). The HET-s PFD comprises two conserved 21-amino acid residue imperfect repeats connected by a poorly conserved 15-amino-acid-long loop (111). Each of these repeats forms one 4.7-Å layer of a stacked β-sheet structure (111, 112). Each repeat comprises four β-strands; the first three β-strands delimit a triangular hydrophobic core, and the fourth protrudes from the core (34, 113) (Fig. 2). At the C-terminal end of the PFD, an aromatic loop folds back into a groove delimited by the third and fourth β-strands to form a semihydrophobic pocket (113). Two asparagine ladders, a frequent motif in amyloids, occur at the beginning of the first and fourth β-strands (N226/N262 and N243/279), and three salt bridges (K229/E265, E234/K270, and R236/E272) stabilize the stacking of the two rungs of β-strands. The inner core is composed exclusively of hydrophobic residues, with the exception of two hydroxyl residues occupying different layers, that can form a hydrogen bond inside the core (T233/S273). Glycine residues occupy the β-arches between the third and the fourth β-strand in both layers. A short C-terminal loop containing two aromatic residues (F286 and W287) folds back onto the fourth β-strand to form a semihydrophobic pocket.

The structure-function relationship in the HET-s PFD was analyzed in detail with site-directed mutagenesis approaches (111, 114, 115). The β-solenoid fold was found to be robust. The majority of the sequence alterations did not affect the global fold or the prion-forming ability. Exceptions are the glycine residues of the β-arches between the third and fourth strand of each rung, whose mutation affects the overall fold and abolishes prion function (114). The different structural elements mentioned above (N-ladders, salt bridges, hydrophobic core, buried polar residues) contribute to various extents to the prion function, β-solenoid fold, and fibril stability and fibril formation rate. Importantly, the C-terminal aromatic loop modulates the prion-forming ability, although this region is not part of the rigid cross-β core of the fold (114). In addition to the solid-state NMR structure, a cryo-electron

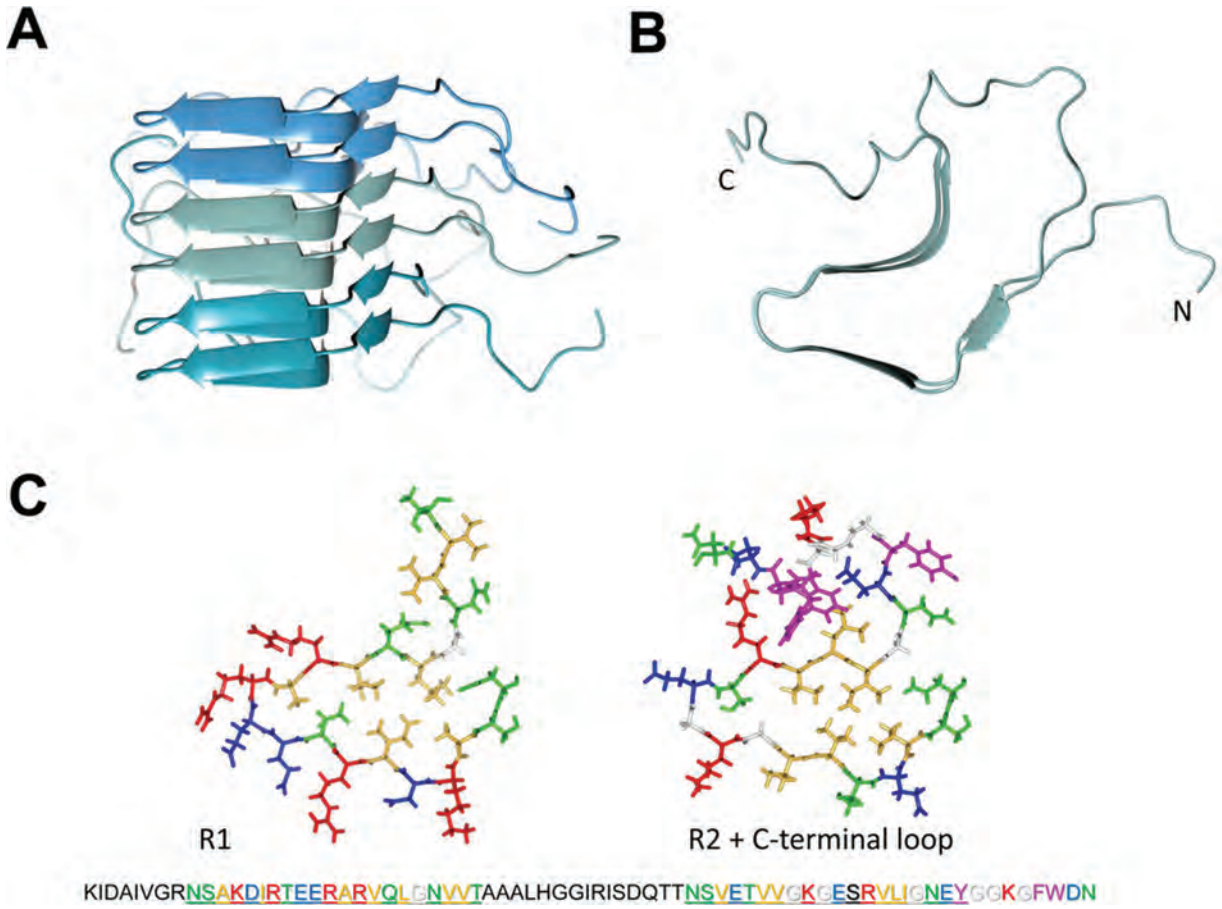


Figure 2 Structure of HET-s prion-forming domain. (**A**) Lateral view of a trimer of HET-s (218–289) in the prion amyloid conformation. Each monomer bears a different color, after pdb 2KJ3. (**B**) View from the fibril axis of one HET-s(218–289) monomer; the N- and C-terminal ends are marked, after pdb 2KJ3. (**C**) Structure of the two individual repeats of HET-s(218–289) marked R1 (position 226 to 246) and R2 (position 262 to 282) as well as the C-terminal semihydrophobic loop (position 283 to 289), after pdb 2KJ3. Amino acids are coded by chemical property (G in light gray, polar in green, hydrophobic in yellow, positively charged in red, negatively charged in blue, and aromatic in magenta). The sequence of HET-s(218–289) is given below with the same color coding in R1, R2 (underlined), and the C-terminal loop.

microscopy structure of the HET-s PFD has also been reported and largely agrees with the solid-state NMR structure (116).

Although no high-resolution structure of a yeast prion is yet available, valuable information on the overall fold of the prion amyloid state of Sup35, Ure2, and Rnq1 was obtained. Several lines of evidence converge to suggest that these yeast prions may adopt an in-register parallel β-sheet structure. Prior to its experimental validation, this organization was already hypothesized based on the experiments mentioned above, showing that shuffled Ure2 and Sup35 PFD sequences (retaining the same amino acid composition) are still able to form prions (41). It was argued that only a parallel in-register β-sheet architecture (and not alternate β-helical or antiparallel or parallel out-of-register models) could explain retention of prion formation with scrambled sequences. Using selectively labeled samples and solid-state NMR, the parallel in-register architecture was supported by dipole-dipole relaxation rates for all three models (101, 117–119). Mass-per-length measurements of prion filaments are also compatible with models in which a single protein molecule occupies one layer of the β-sheet structure (that is, 4.7 Å) (85, 120, 121). Collectively, these results are compatible with the structural model proposed by Kajava and coworkers (122) and can account for strain

variation. They stand in contrast to an alternative β-helical model for Sup35 based on chemical cross-linking approaches (123). The highly degenerate nature of Sup35 fibers complicates interpretation of these data, and a full atomic-level understanding of yeast prion structure will likely require the application of new technologies to the problem.

CONCLUSION

Our understanding of prion biology is built on the rich curiosity of researchers investigating undefined epigenetic phenotypes in fungi beginning over 50 years ago. Their persistence has led to a wealth of knowledge that now impacts our understanding of biological complexity and human health in a multitude of ways. While many incredible discoveries have been made thus far, these systems lend themselves to additional advances relating to epigenetics, the biology of complex phenotypes, prion structure and replication, prion diversity, the effects of intracellular and extracellular modulators, and the impact of protein-based elements on organismal fitness and survival.

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Repeat-Induced Point Mutation and Other Genome Defense Mechanisms in Fungi

33

Eugene Gladyshev

DEFENDING THE GENOME AGAINST MOBILE DNA

Mobile DNA, comprising both active and decaying copies of transposable elements (TEs), is present in nearly all living organisms. Although fungal genomes tend to be significantly smaller than the genomes of plants and animals, they still can vary dramatically with respect to their TE loads (1). While TEs have been proposed to provide some beneficial functions to their hosts, e.g., by promoting genetic diversity and accelerating adaptive evolution (2–5), their overall impact is considered deleterious (6). Insertional mutagenesis, gene misexpression, and genome instability represent some well-known examples of the deleterious effects associated with TEs. Importantly, by being able to move between vertical genetic lineages, TEs can still proliferate in a population of sexually reproducing individuals despite causing substantial fitness defects (6).

The ability of TEs to multiply exponentially in the host genome is opposed by the host genome defense systems. Known defense mechanisms differ significantly with respect to their specific modes of TE recognition and suppression, yet all of them face the same basic challenge of being able to recognize TEs among many other potential genomic targets. Specific recognizable features of TEs include (i) frequently aberrant modes of replication and transcription of TEs, (ii) the presence of multiple TE copies in the host genome, and (iii) the occurrence of polymorphic TE insertion sites in the host population. Once detected, TEs can be silenced by a number of molecular approaches. The canonical examples of transcriptional and cotranscriptional silencing include RNA-directed methylation of DNA (cytosines) in plants (7) and of H3K9 in the *Drosophila* germ line (8), as well as various cotranscriptional modalities involving RNA processing and quality control (9–14). TE-encoded RNA can also be restricted posttranscriptionally by RNA interference (RNAi) and RNA editing. In addition to these transcription-related processes, TEs can also be inactivated by the physical elimination of their DNA, as it occurs during chromatin diminution in selected eukaryotic groups including ciliates, foraminifera, and some animals (15, 16).

GENOME DEFENSE MECHANISMS IN FUNGI

In fungi, TEs are opposed by several genome defense mechanisms including repeat-induced point mutation (RIP) and methylation induced premeiotically (MIP), meiotic silencing by unpaired DNA (MSUD), sex-induced silencing (SIS), somatic quelling (cosuppression), and several cotranscriptional RNA surveillance processes (exemplified by spliceosome-coupled and nuclear RNAi [SCANR]).

Cotranscriptional RNA Surveillance Processes: SCANR

TEs are often associated with aberrant transcription, which is recognized by cotranscriptional RNA surveillance and which triggers silencing either by a localized assembly of heterochromatin or by an RNAi response (9–14). Because cotranscriptional RNA surveillance can act on individual TE copies in vegetative cells, it provides the first line of defense and allows the host to

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silence TEs that do not normally produce double-stranded RNA (dsRNA) and do not exist as tandem repeat arrays. One such mechanism was recently described in the basidiomycete *Cryptococcus neoformans* by Phillip Dumesic, Hiten Madhani, and colleagues (13), who discovered and characterized the SCANR complex. Their results led to a kinetic model of post-transcriptional silencing, by which the presence of sub-optimal introns in TE-encoded pre-mRNAs promotes inefficient splicing and induces spliceosome stalling, thus providing SCANR with sufficient time to initiate the RNAi (13).

Somatic Quelling (Cosuppression)

The phenomenon of somatic quelling was first described by Nicoletta Romano and Giuseppe Macino in *Neurospora crassa* (17). Generally known as cosuppression in plants and animals, somatic quelling represents a paradigmatic example of an RNAi-mediated posttranscriptional defense mechanism. In somatic quelling, small interfering RNAs are produced specifically from chromosomal loci that contain repetitive DNA (18). In addition to the canonical RNAi factors (the RNA-dependent RNA polymerase QDE-1, the Argonaute protein QDE-2, and Dicer-like proteins DCL-1 and DCL-2), somatic quelling also requires RPA (replication protein A), QDE-3 (RecQ/SGS1 DNA helicase), and the key recombination factors Rad51, Rad52, and Rad54 (19). In light of these and other results, a model was proposed by which single-stranded DNA intermediates formed during aberrant recombinational DNA repair can serve as templates for the production of primary single-stranded "aberrant" RNAs using the DNA-dependent RNA polymerase activity of QDE-1. Single-stranded aberrant RNAs would be converted to dsRNAs by the second, RNA-dependent RNA polymerase activity of QDE-1. In their turn, dsRNAs would then be processed into mature small RNAs (qiRNAs) associated with the Argonaute protein QDE-2 (19).

Interestingly, in *N. crassa*, repetitive transgenes that can potentially produce qiRNAs may also become associated with heterochromatic H3K9me3 and DNA methylation by a poorly understood process that does not involve RNAi (20–23). It is tempting to speculate that both RNAi-dependent and RNAi-independent mechanisms cooperate to silence expression of deleterious repetitive elements in vegetatively growing cells.

Meiotic Silencing

The phenomenon by which "unpairable" chromosomal segments become reversibly silenced in meiosis was first discovered by Robert Metzenberg and colleagues in *N. crassa* (24, 25). Together, their pioneering studies demonstrated that the presence of a gene-sized DNA segment on only one of the two homologous (parental) chromosomes could trigger potent yet transient silencing of all its copies (both pairable and unpairable) in the meiotic cell. This phenomenon was named meiotic silencing by unpaired DNA (MSUD) (25). In *N. crassa*, MSUD acts as a *bona fide* defense mechanism against TEs (26) and meiotic-driven elements called spore killers (27). This silencing process can be triggered by a chromosomal segment of unpairable DNA as short as 1 to 2 kbp, and increasing the segment length to 4 to 5 kbp leads to a much stronger effect (27). In its scope of detected targets, MSUD appears to be complementary to RIP (below). Both processes operate during the sexual cycle, yet while RIP specifically inactivates TEs that exist in a given parental genome as multiple copies, MSUD can detect only one TE copy as long as this copy is present in only one parental genome.

Processes analogous to MSUD were also described in animals (28). The earliest observations of meiotic silencing in mammalian males pertained to the phenomenon of meiotic sex chromosome inactivation, in which the XY bivalent becomes progressively condensed and associated with repressive chromatin modifications during the first meiotic prophase (28). Subsequent studies by several groups showed that meiotic silencing in animals was not restricted to the sex chromosomes (29–32). Because much larger chromosomal regions were required to trigger silencing (as compared with MSUD in *N. crassa*), the animal process was named "meiotic silencing of unsynapsed chromatin." In both animals and fungi, meiotic silencing is activated in prophase I, when pairs of homologous chromosomes become progressively condensed and synapsed (28). Whereas meiotic silencing in animals operates primarily at the transcriptional level and involves changes in the pattern of histone modifications over large chromosomal domains, meiotic silencing in fungi is mediated at the posttranscriptional level by RNAi (27, 33).

How MSUD can recognize unpairable DNA remains a great mystery. Intriguingly, MSUD fails to detect two identical chromosomal segments as unpairable if these segments are present on homologous chromosomes at nonallelic positions separated by a few thousand base pairs (34). This result suggests that as long as two DNA segments with the same or nearly the same nucleotide sequences have an opportunity to interact with one another in the context of the juxtaposed homologous chromosomes, they are likely to be recognized as homologous and thus suppress the MSUD response. Once the distance between such nonallelic segments is

increased, the probability of their interaction decreases, triggering MSUD (34). These important results hint at a possibility that relatively short chromosomal segments behave as spatially constrained yet quasi-autonomous entities (27). In this case, each segment may be evaluated for the presence of a pairable partner independently from its neighboring genomic sequences. The nature of the constraint that limits interactions between homologous segments present at widely separated nonallelic positions is unknown. Yet it has been speculated that it might be imposed by the basic organization of meiotic chromosomes that involves chromatin loops tethered to the chromosomal axis (27). In light of these considerations, MSUD may emerge as a powerful new tool for probing the structure of condensed meiotic chromosomes.

The majority of factors required for MSUD in *N. crassa* (including an RNA-dependent RNA polymerase SAD-1, the Dicer-like protein DCL-1, an Argonaute protein SMS-2, and an RNA helicase SAD-3) are concentrated in the perinuclear space and therefore are physically removed from the presumptive site of homology recognition involving meiotic chromosomes (35, 36). The mechanism by which the presence of a locally unpairable DNA segment in the nucleus triggers the RNAi response in the perinuclear space remains deeply mysterious (27, 33). Recent work by Tom Hammond, Patrick Shiu, and colleagues in *N. crassa* identified SAD-6 as the first conserved nuclear factor involved in MSUD (34). SAD-6 belongs to a SWI/SNF subfamily of chromatin-remodeling proteins that also includes ATRX (34), yet because MSUD still occurs in the absence of SAD-6, this factor could play a redundant or secondary role in meiotic silencing.

Sex-Induced Silencing (SIS)

Joseph Heitman and colleagues discovered SIS in *C. neoformans* (37). In SIS, tandem repeat arrays are detected and silenced by RNAi during the premeiotic stage of the sexual cycle. While the same tandem repeat array can trigger an RNAi response in *C. neoformans* prior to entering the sexual phase, the efficiency of this somatic process appears to be much lower than in SIS (37, 38). Interestingly but not unexpectedly, all the canonical RNAi components required for SIS (RNA-dependent RNA polymerase as well as Dicer and Argonaute proteins) become transcriptionally upregulated specifically during the sexual phase when SIS occurs (37). Once initiated, SIS can be propagated during vegetative growth for many generations in the absence of any apparent repressive epigenetic modifications that are normally associated with heritable silencing in fungi (37), suggesting that RNAi alone can permit stable inheritance of the silenced state.

Intriguingly, SIS in the basidiomycete *C. neoformans* appears to share some basic features with both RIP (below) and somatic quelling (above) in ascomycete fungi. For example, similar to somatic quelling, SIS involves RNAi and is triggered by the presence of tandem repeat arrays. On the other hand, similar to RIP, SIS is strongly activated during the premeiotic phase. In general, the premeiotic phase appears to play a special role in both basidiomycetes and ascomycetes, perhaps by allowing the parental genomes to be thoroughly searched for TEs before these genomes become mixed, recombined, and inherited by the next generation.

RIP Mutation

Soon after transformation techniques were developed in *N. crassa* (39), it was noticed that transgenic repetitive DNA became unstable specifically during the sexual phase in this organism (40). At about the same time, Eric Selker and Judith Stevens reported strong cytosine-to-thymine (C-to-T) mutation and concomitant methylation of nearly all remaining cytosines in a naturally occurring tandem duplication of a 5S RNA gene (41). A peculiar association of strong C5-cytosine methylation with repeated DNA encouraged the idea of a causal relationship, whereby the presence of repeats provided a signal for cytosine methylation and mutation (41). Pioneering work by Selker and colleagues demonstrated that the repetitive nature of DNA indeed provided such a signal in a process named repeat-induced point (RIP) mutation. Subsequent work by Selker and colleagues defined some key properties of RIP in *N. crassa* (42–44). Specifically, it was shown that RIP

- Occurs specifically in haploid parental nuclei that continue to divide by mitosis in preparation for karyogamy and ensuing meiosis
- Detects duplications of chromosomal DNA above a certain length threshold (~0.4 kbp), irrespective of their transcriptional status, origin, and relative as well as absolute positions in the genome (although closely positioned repeats are detected much more readily than widely separated repeats)
- Mutates cytosines on both strands of each DNA duplex in a pairwise fashion (e.g., preferentially affecting none or both repeated sequences)
- Occasionally spreads from duplicated sequences into neighboring nonrepetitive regions
- Remains unaffected in *mei-2* crosses that exhibit a strong defect in meiotic pairing of homologous chromosomes

Since its discovery in *N. crassa* nearly 30 years ago, the existence of RIP has been experimentally confirmed in several ascomycete species including *Magnaporthe oryzae*, *Podospora anserina*, *Leptosphaeria maculans*, and *Fusarium graminearum* (45). Further bioinformatic analysis of the sequenced genomes of filamentous fungi revealed widespread signatures of RIP-like mutation (46–48). Intriguingly, elevated C-to-T mutation of repetitive sequences was also reported in several basidiomycete fungi, suggesting that the evolutionary appearance of RIP had preceded the separation of Ascomycota and Basidiomycota (49). Yet, while being a potentially ancient phenomenon, RIP does not seem to be strongly conserved even between related species. For example, while RIP is particularly strong in *N. crassa*, it cannot be detected at all in its relative *Sordaria macrospora* (50).

Methylation Induced Premeiotically (MIP)

Soon after the discovery of RIP, Christophe Goyon and Godeleine Faugeron (51) provided the first account of a related premeiotic silencing phenomenon that occurred in a distant relative of *N. crassa*, the ascomycete *Ascobolus immersus*. Unlike RIP mutations, the silenced phenotype could be spontaneously reversed during subsequent vegetative growth by the loss of cytosine methylation (51). These and other pioneering observations suggested that in *A. immersus* premeiotic silencing was mediated by cytosine methylation alone and did not involve permanent changes in the DNA sequence. By analogy with RIP, this process was named methylation-induced premeiotically (MIP) (52).

Further work by Jean-Luc Rossignol, Godeleine Faugeron, and colleagues provided strong evidence that both MIP and RIP represent two manifestations of the same basic process (43, 52, 53). Just like RIP, MIP detects gene-sized duplications of chromosomal DNA involving either native or foreign sequences (54). For a duplication of a given length, closely positioned repeats were always detected more efficiently than widely separated repeats. Similarly to RIP (55), MIP also appears to sense repeats in a pairwise fashion: if three repeat copies were present in the same nucleus, normally two or, less frequently, three copies would become inactivated, whereas silencing of only one copy was rarely observed (54). Further analysis of the homology length requirements for MIP showed that MIP could detect closely positioned repeats as short as 317 bp (56) and widely separated repeats as short as 1.2 kbp (57). Very similar length requirements had been reported for RIP (42–44), and in both processes cytosine modifications occurred primarily over the extent of the repeated region.

CYTOSINE METHYLTRANSFERASES Masc1 AND RID AS THE CANONICAL MEDIATORS OF MIP AND RIP

The discovery of Masc1 (methyltransferase from ascobolus 1) by Fabienne Malagnac and colleagues offered the first mechanistic insight into the nature of MIP and, consequently, RIP (58). Masc1 was identified by cloning a short fragment of the *masc1* gene using a degenerate PCR approach. Because Masc1 appeared to play an essential role during the sexual stage in *A. immersus*, its role in MIP could only be examined in heterozygous conditions. In those situations, MIP could still be reduced by more than 2 orders of magnitude, thus showing a strong dependence on Masc1. Interestingly, cytosine methylation was still maintained in postmeiotic cells carrying the silenced copy of *masc1* (58). This finding suggested that another, Masc1-independent mechanism was responsible for propagating cytosine methylation induced by Masc1 in the premeiotic cells. Because Masc1 played an essential role during the sexual cycle and because it specifically affected DNA repeats, it was proposed to regulate timely expression of developmental genes associated with naturally occurring instances of repetitive DNA (58). By this proposal, MIP could fulfill both the genome-defense and regulatory functions.

N. crassa has only one Masc1 homolog, and studies by Michael Freitag, Eric Selker, and colleagues showed that it also played a critical role in RIP (59). Because of its essential role in RIP in *N. crassa*, the protein was named "RIP defective" (RID). Unlike Masc1, the absence of RID does not result in any apparent defect during the sexual cycle because homozygous *rid/* crosses develop normally and still produce large numbers of viable progeny spores (59). This finding suggests that, unlike Masc1 in *A. immersus*, RID may play a more specialized role in *N. crassa*, perhaps restricted only to its genome defense function during RIP. One RID homolog is also present in *S. macrospora*, which does not appear to have active RIP but which still contains a surprisingly low number of TEs, hinting at a possibility that a related RID-dependent genome defense mechanism could be involved (50).

Subsequently, Masc1/RID homologs were identified in nearly all ascomycetes, where they appear to play a conserved role during the sexual stage (60). For example, another RID homolog, DmtA, was recently implicated in regulation of asexual development and secondary metabolism in the ascomycete *Aspergillus flavus* (61). Among all known representatives of this ancient clade of putative cytosine methyltransferases, its founding member

Masc1 still appears to have the most prototypical structure. The predicted amino acid sequence of Masc1 includes a compact catalytic domain of about 300 amino acids and an N-terminal region of about 200 amino acids. *Neurospora* RID, in addition to the catalytic and the N-terminal regions, also includes a long C-terminal extension of 260 amino acids that is also present in the homolog of RID in *S. macrospora* (50). The N-terminal regions of both Masc1/RID can be fully aligned with the corresponding region of the mammalian DNMT1 and contain a weak signature of a bromo-adjacent homology domain (62). Interestingly, Masc1/RID proteins comprise a very ancient group of proteins that emerged long before the separation of animals, plants, and fungi (58, 63), yet these proteins can only be found currently in the subphylum of Pezizomycotina.

Intriguingly, all Masc1/RID proteins appear to have the structure of motif VI that deviates from the structure of motif VI in all other prokaryotic and eukaryotic C5-cytosine methyltransferases (63). The canonical amino acid sequence of motif VI contains the ENV (glutamate-asparagine-valine) triad. The glutamate residue is absolutely essential for catalysis (64) and it is also conserved in the Masc1/RID proteins (Fig. 1). The adjacent asparagine residue (ENV) interacts with the proline residue of the catalytic triad PCQ (in motif IV) and thus plays an important architectural role by controlling the positions of the ENV and PCQ triads with respect to one another (65, 66). The valine residue of ENV also appears to be functionally important, because changing it to alanine in the model prokaryotic methyltransferase M.HhaI inactivates the enzyme (67).

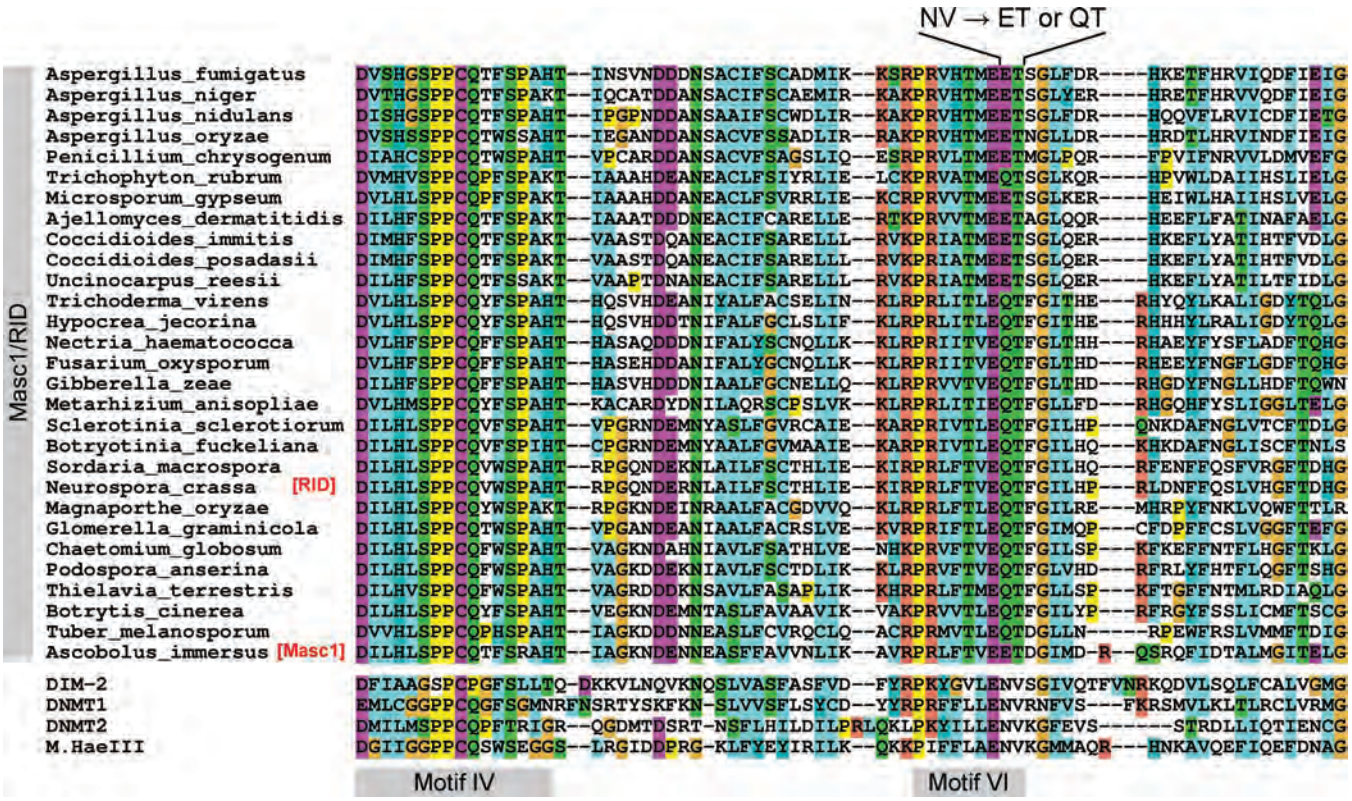


Figure 1 The structure of motif VI in Masc1/RID proteins is not canonical. The canonical motif VI contains the absolutely conserved NV diad (asparagine-valine). This diad is present in all C5-cytosine methyltransferases except Masc1/RID. The asparagine residue of NV physically interacts with the proline residue of the catalytic triad PCQ (in motif IV) and thus plays a critical role by controlling the positions of these segments with respect to one another in the native structure of the protein. The valine residue of NV is also functionally important, because its substitution for alanine is known to inactivate the catalytic activity of M.HhaI. Yet in all Masc1/RID proteins the NV diad is replaced with either QT (e.g., in *Neurospora* RID) or ET (e.g., in *Ascobolus* Masc1), hinting at the possibility that Masc1/RID proteins might have unique catalytic and/or substrate requirements.

Yet in all Masc1/RID proteins, the NV diad is replaced with either QT (e.g., in *Neurospora* RID) or ET (e.g., in *Ascobolus* Masc1), hinting at the possibility that these enzymes might have evolved unique catalytic and/or substrate requirements. Interestingly, the catalytic activity of the purified Masc1 protein could not be detected *in vitro* (58), thus further supporting that idea that Masc1/RID proteins may be different from the other C5-cytosine methyltransferases and/or may require auxiliary factors for catalysis.

UNDERSTANDING THE HOMOLOGY REQUIREMENTS FOR RIP

The apparently pairwise nature of repeat mutation during RIP (55, 68) suggested that the process of repeat recognition might also involve a pairwise comparison of DNA sequences. The discovery that RIP proceeded normally in the absence of SPO11 and MEI-3 (the only RecA homolog in *Neurospora*) excluded the most obvious recombination-mediated pairing mechanism (69, 70), yet it did not provide any new leads toward understanding the nature of the homology search process during RIP.

A Quantitative Measure of RIP Mutation

Additional insights into the mechanism of repeat recognition for RIP were obtained by systematically investigating the homology requirements of this process. As a starting point, a sensitive assay for RIP was developed in *N. crassa*, by which the propensity of engineered repeats to induce RIP mutation could be accurately quantified (69, 70). By this approach, each tested repeat construct was first integrated into a standard chromosomal locus of a standard *Neurospora* strain. It was then exposed to RIP during the sexual phase, while the occurrence of C-to-T (and G-to-A) mutations was revealed by sequencing the entire construct in a sample of randomly selected progeny spore clones (62). This quantitative approach was made possible by some specific features of *Neurospora* reproductive biology, by which individual DNA molecules were subjected to RIP over the course of several days, while the final molecular product of RIP was released from the perithecium as a spore clone that could be recovered by PCR and sequenced. A bioinformatic pipeline was developed to detect and analyze the occurrence of C-to-T and G-to-A mutations along each individual RIP product. By this approach, new aspects of RIP could be revealed by evaluating the dependence of RIP mutation on the configuration and homology relationships of strategically designed repeat units.

The assay also incorporated a number of features that permitted reliable quantitative readout of RIP (62). First, RIP activity was expressed and analyzed as the total number of mutations. Second, RIP activity was maximized by assaying pairs of closely positioned repeats. Third, the analysis was focused on meiotic spores produced relatively late in the cross, because such spores were associated with intrinsically higher levels of RIP. Fourth, noting that RIP mutation could vary substantially between different strains and between spore clones in the same cross, the extraneous variation in RIP activity was minimized by (i) integrating all repeat constructs into the same locus (*csr-1*) of the same strain (FGSC#9720) and (ii) crossing each transformant (as a male parent) to the same female strain (FGSC#4200). This particular pair of strains (FGSC#4200 × FGSC#9720) was chosen because they consistently produced a large number of viable progeny spores, thus providing an adequate statistical population from which individual RIP products could be sampled. In this case, per-spore variability in RIP levels could be addressed by sampling a substantial number of spores from these large populations (62). Finally, the variation in RIP levels between experiments involving different repeat constructs was further reduced by analyzing constructs in which one (and always the same) repeat copy was held constant, while the other repeat copy could be manipulated as desired (with respect both to its length and orientation as well as to its base pair sequence).

Quantifying RIP Response to Perfect Homologies of Varying Length

During RIP, the presence of homologous DNA sequences results in characteristic mutations over these same sequences. To understand the basic relationship between the length of homology and the number of ensuing mutations, the response of RIP to repeats of graded length (ranging from 155 to 802 bp) was measured (69). It was discovered that 155 bp of perfect homology could trigger very low but still detectable levels of RIP. The fact that this homology length was significantly shorter than the previously published threshold (71) suggested that the assay indeed provided a very sensitive readout of RIP activity. Intriguingly, further analysis showed that the mean number of RIP mutations over a region of interest shared between all the constructs was strongly proportional to the fourth power of homology length, specifically for repeats ranging between 220 and 520 bp (69). This finding suggested that the number of mutations could be used as a sensitive and accurate measure of DNA homology in the length range amenable to experimentation.

Recognition of Weak Interspersed Homologies by RIP

Recognition of partially homologous repeats by RIP in *N. crassa* was previously examined by Selker and colleagues (72). In this pioneering work, instances of partial homology were generated by the process RIP mutation itself. It was discovered that new cycles of RIP could be triggered by previously mutated repeats that retained more than 80% of sequence identity (72). This result suggested that the mechanism of repeat recognition for RIP required substantial DNA homology. It was consistent with the idea that DNA homology was sensed in the context of an uninterrupted alignment of single strands, because that is the case for the canonical, recombination-mediated homology recognition.

Intriguingly, recent studies revealed that, despite being very sensitive to the overall amount of homology, RIP could easily "overlook" substantial interruptions in homology if such interruptions occurred in the middle of the repeated sequence (69). This result hinted at a possibility that the mechanism of homology recognition for RIP did not necessarily require a continuous sequence alignment. The next question raised by these results was the following: if RIP could readily detect trivial cases of interrupted homology, what would be the weakest (or the atomic) instance of interrupted homology that could still be recognized by RIP?

The advent of efficient DNA synthesis technologies permitted systematic analysis of the homology requirements for RIP (69). Because RIP was particularly sensitive to the overall amount of homology in the range of 220 to 520 bp (69), synthetic DNA segments of the relevant size could be made rapidly and inexpensively, and thus many such segments could be tested and analyzed for their ability to promote RIP (62, 69). By this approach, it became clear that homology patterns with overall sequence identity much less than 80% could still be detected by RIP. Systematic examination of many such patterns suggested that only specific homology configurations could be recognized. All "active" homology patterns consisted of short homologous units (3 bp or more) that had to be spaced at regular intervals of 11 or 12 bp along the overall extent of homology (69, 70). Figure 2 provides an example of one such pattern, in which homologous units of 4 bp are interspersed with the periodicity of 11 bp along the total length of 500 bp. Importantly, for this process to work, DNA segments had to be coaligned along their length. This result suggested that homology was detected between coaligned and rigid objects. When considered together with the periodicity requirement, these results suggested that these rigid objects could be the double helices of DNA. Thus, a model was proposed by which homologous DNA molecules could become engaged in multiple, interspersed, triplet-mediated, cooperative interactions taking place over the length of several hundred base pairs. Additional results suggested that RIP mutation could be decoupled from the process of homology recognition, suggesting a possibility in which the DNA-modifying activities were recruited to DNA segments after they have been defined as homologous.

THE HETEROCHROMATIN-RELATED PATHWAY OF RIP

The Universal Phenomenon of Heterochromatic Silencing of Repetitive DNA

In the genomes of nearly all eukaryotic organisms, highly repetitive DNA sequences are organized in the form of constitutive heterochromatin, a specialized state of chromatin that remains condensed during interphase and associated with conserved repressive epigenetic modifications, such as di- and trimethylated lysine-9 residues of histone H3 (H3K9me2/3). The apparent mechanistic connection between the repetitive nature of the genetic material and its heterochromatic nature was noted by Guido Pontecorvo in 1944 when he wrote, "a heterochromatic segment should arise every time that a minute euchromatic region undergoes repeated reduplications in the genotype and the replicas remain adjacent to each other on the chromosome" (73).

The idea that the heterochromatic state can be induced by homologous interactions between repeat elements was further advanced by Douglas Dorer and Steven Henikoff in 1944 (74). Working with *Drosophila melanogaster*, the authors generated a series of tandemly repeated *mini-white* transgenes and discovered that the level silencing of these repetitive transgenes was dependent on the number of repeat copies. Based on these and other results, it was suggested that heterochromatin proteins could specifically recognize the higher-order chromatin structures produced by the stable, homology-dependent association of nearby repeat copies (74). By this proposal, and in accord with the original idea of Pontecorvo, heterochromatin could form on repetitive DNA of any particular sequence, simply because of its ability to pair with itself and produce higher-order structures. The authors also noted that such homology-directed pairing could underlie silencing of long tracts of simple trinucleotide repeats (74). Notably, this same idea, by which the expanded trinucleotide

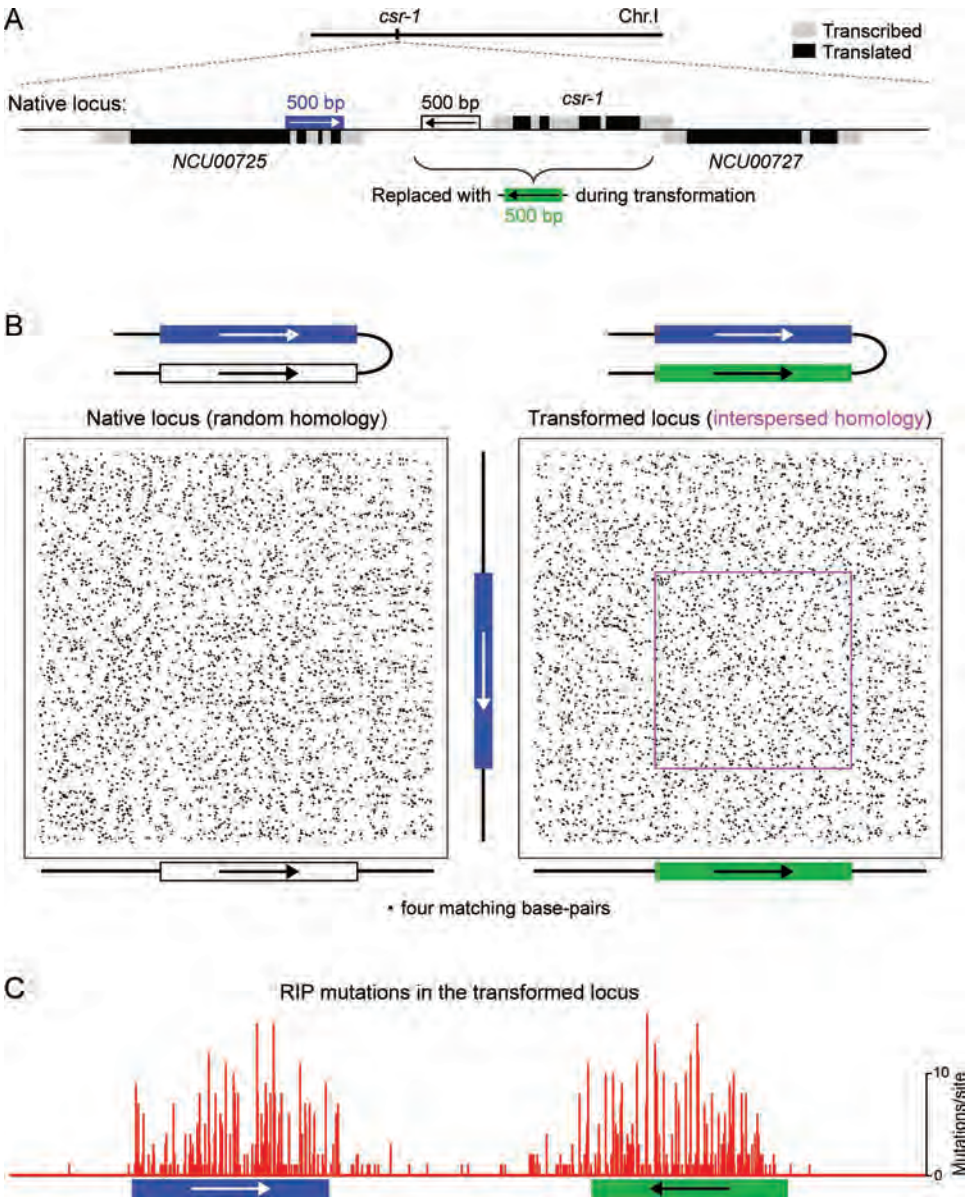


Figure 2 Recognition of interspersed homology during RIP in *N. crassa*. This assay detects and quantifies the occurrence of RIP mutations in response to engineered DNA repeats. Instances of DNA homology are created between two short segments of chromosomal DNA, one of which is normally represented by an endogenous sequence, while the sequence and orientation of the other segment can be manipulated as desired. In this situation, the number of RIP mutations provides a very sensitive readout of DNA homology perceived by the recombination-independent mechanism of repeat recognition for RIP. (**A**) Weak interspersed homology is formed between the endogenous 500-bp segment (blue) and a synthetic DNA segment (green) integrated at a nearby position as the replacement of the *cyclosporin-resistant-1* (*csr-1*) gene. This particular pattern involves 4-bp units of homology spaced with the periodicity of 11 bp and exists between "repeat units" in the inverted orientation. (**B**) Pairwise sequence comparisons showing all matches of 4 bp long. Two situations are presented: random homology (left panel) and interspersed homology (right panel). No cryptic homology can be seen except the intended pattern of weak interspersed homology (magenta box). (**C**) The occurrence of mutations induced by weak interspersed homology. Seventy progeny spores from the "XKO" cross (70), which had been previously found to contain at least one RIP mutation, were reanalyzed by sequencing of an additional 255 bp in the "left" flank of the construct (corresponding to the single-copy coding/translated sequence of *NCU00725*).

arrays could act as two closely positioned repeat copies, was also proposed by Goyon, Rossignol, and colleagues in the context of their studies on MIP (56).

Subsequent studies of *D. melanogaster* by Sarah Elgin and colleagues, using some of the original *mini-white* arrays of Dorer and Henikoff, showed that the heterochromatic state of these repeats was dependent on the presence of functional RNAi (75). Furthermore, around the same time, the key role of RNAi in heterochromatic silencing was also demonstrated in fission yeast (76, 77) and in plants (78), suggesting that the presence of tandem repeats *per se* was not sufficient, at least in some situations, to program the formation of functional heterochromatin.

The Canonical Heterochromatin Pathway in *Neurospora*

In *N. crassa*, the bulk of H3K9me3 and C5-cytosine methylation can be found in the context of AT-rich DNA that was previously mutated by RIP (33). All mechanistic aspects of this pathway were elucidated largely by the work of Selker and colleagues (33). In brief, the canonical pathway starts with RIP generating AT-rich DNA in premeiotic cells, which then provides a signal for the recruitment of heterochromatin factors in vegetative cells (33). The key heterochromatin mark, H3K9me3, is catalyzed by the conserved SUV39 methyltransferase DIM-5 (33). H3K9me3 is recognized by the *Neurospora* HP1, which directly recruits the cytosine methyltransferase DIM-2 to DNA (33). This pathway involves some additional factors (33), and in some situations, it can deviate from its canonical form (79).

Both RIP and repeat-induced heterochromatic silencing may be considered as serving the same purpose of suppressing repetitive DNA. Yet in *Neurospora*, the relationship between RIP (which occurs in premeiotic cells) and heterochromatic silencing (which occurs in vegetative cells) presented a puzzle (43). It was known for a long time that in *N. crassa*, newly introduced highly repetitive transgenes could become associated with H3K9me3 and cytosine methylation in vegetative cells, prior to RIP and in the absence of RNAi (20–23, 80, 81). These results, which were obtained by several independent research groups, supported the idea that the presence of repetitive DNA could induce heterochromatin directly, in accord with the original proposal by Pontecorvo (73) and Dorer and Henikoff (74).

Neurospora RIP Can Be Mediated by the Heterochromatin Pathway

Recently, it was discovered that *Neurospora* RIP could occur in the absence of RID, suggesting that (i) RID was not essential for the process of repeat recognition *per se*, and (ii) RID was not the only mediator of RIP mutation (82). Surprisingly, it was further discovered that all RID-independent RIP required DIM-2 and the catalytic activity of DIM-5 (82). Further investigation revealed that the DIM-5/DIM-2 pathway of RIP (i) was triggered specifically by the presence of repetitive sequences, (ii) could respond to repeats present at closely positioned as well as widely separated genomic positions, and (iii) mutated repeats that had never been previously subjected to RIP. Importantly, the DIM-5/DIM-2-mediated RIP could also be activated by weak interspersed homologies, in which 3-bp or 4-bp units of homology were arrayed with the 11-bp periodicity, supporting the idea that both the RID- and DIM-5/DIM-2-dependent pathways of RIP are triggered by the same homology signal. In every case examined, DIM-5/DIM-2-mediated mutations typically spread from the repetitive elements into the neighboring single-copy regions, whereas RID-mediated mutations are largely restricted to the repeats *per se* (82).

A Putative Two-Step Mechanism of RIP Mutation

The original discovery of RID as a principal mediator of RIP (59) presented a puzzle: while RIP involves C-to-T mutation, RID resembles a canonical C5-cytosine methyltransferase. Although the predicted catalytic site of RID differs from all other C5-cytosine methyltransferases (above), so does the active site of Masc1, which mediates cytosine methylation, without any sign of mutation (58). Thus, it was originally proposed that the modification of cytosines during RIP might occur as a two-step process, in which C5-methylation by RID would be followed by N4-deamination of the C5-methylated cytosine by an unrelated enzymatic activity (59). An alternative proposal was also put forward by which RID alone could mediate C-to-T mutation, via modulation of its catalytic activity (59). This hypothesis was supported by earlier studies (83, 84), which implicated canonical cytosine methyltransferases (such as the prokaryotic site-specific methyltransferase M. HpaII) in catalyzed deamination of cytosines under conditions of extremely low levels of the methyl group donor *S*-adenosylmethionine.

The finding that DIM-2 can mediate RIP provides additional support for the two-step mechanism. In vegetative cells, DIM-2 normally catalyzes cytosine methylation in all dinucleotide contexts (85). Yet DIM-2-dependent RIP exhibits a very strong bias for CpA dinucleotides (82). While it is formally possible that the intrinsic substrate preference of DIM-2 in premeiotic

versus vegetative cells is different, the strong CpA bias of DIM-2-mediated RIP mutation argues in favor of the presence of two separate enzymatic activities for catalyzing cytosine methylation and deamination. By this model, repeat-induced cytosine methylation (by either DIM-2 or RID) would be converted to mutation (5meC → T) by a deaminase activity that is specifically present during the premeiotic (germ line) phase. Interestingly, novel deaminase activities (involved in A → I editing of RNA) were recently identified in *Neurospora* and some other filamentous fungi (86, 87). These activities are only present during the sexual stage, as would be expected from the hypothesized deaminases involved in RIP. Nevertheless, it is important to note that in *Neurospora* vegetative cells, DIM-2 can also methylate coding, nonrepetitive parts of the genome (88). If cytosine methylation of genic regions also takes place in premeiotic cells, then the proposed two-step mechanism would further require the deamination step to be homology-dependent.

Hypothesis: Nucleation of Heterochromatin by Homologous dsDNA/dsDNA Interactions

The newly discovered role of the DIM-5/DIM-2 pathway in RIP suggests that homologous interactions between coaligned double-stranded segments of chromosomal DNA, which were previously proposed to mediate repeat recognition for RIP (69), can provide a specific signal for the recruitment of these heterochromatin factors. It has been known for a long time that in *N. crassa* the same pathway can mediate RNAi-independent heterochromatic silencing of repetitive transgenes in vegetative cells. Taken together, these results suggest that the heterochromatic silencing of transgenes in vegetative cells can also be programmed by interactions between homologous dsDNA molecules. By this general model, the occurrence of homologous dsDNA/dsDNA interactions provides a physical signature of DNA homology.

In *Neurospora* vegetative cells, homology-directed heterochromatic silencing could play a backup role and/or could come into play in situations where repetitive DNA is newly introduced prior to RIP. Intriguingly, the structure and function of DIM-5 are conserved in other fungi, as well as in plants and animals, hinting at the possibility that homology-directed heterochromatic silencing may be a general phenomenon. This homology-directed process can function in combination with other, more canonical silencing mechanisms. In this case, transient H3K9me3 marks, induced by dsDNA/dsDNA interactions, can be amplified and propagated by additional mechanisms involving site-specific DNA binding proteins, such as Zeste (74, 89), or RNA (74). Because the proposed pairwise dsDNA/dsDNA interactions involving nearby repeats can occur in several successive rounds, and because multiple independent interactions may take place over a long repeat array at the same time, this process could be particularly efficient over large repetitive regions, such as those found in (peri)centromeric and (sub)telomeric regions of chromosomes.

CONCLUDING REMARKS

Because of their ability to move between genetic lineages and proliferate exponentially, TEs pose an existential threat to genome stability. The evolutionary race between TEs and the host genome defense systems produced a large repertoire of solutions to the basic problem of keeping the mobile repetitive DNA in check. Most of these solutions are not absolute, and none of them (with the exception of RIP in *N. crassa*) can neutralize all TEs at once. Yet the emergence of especially strong RIP in *N. crassa* has come with a cost, because this organism appears to lack gene duplications, a major source of evolutionary novelty (90).

The genomes of most eukaryotes contain large amounts of TEs and other forms of repetitive DNA that are normally silenced in the form of constitutive heterochromatin. The newly discovered role of the heterochromatic pathway in *Neurospora* RIP suggests that these two phenomena may be evolutionarily related. In this case, RIP may simply represent a specialized heterochromatin-related process that terminates in C-to-T mutation instead of cytosine methylation. Previous studies have indicated that the mechanism of repeat recognition for RIP may involve interactions between coaligned dsDNA molecules. A possibility now emerges that such direct dsDNA/dsDNA interactions may underlie a much more general phenomenon of heterochromatic silencing of repetitive DNA, possibly representing a primordial genome defense mechanism. Further analysis of homology-directed silencing phenomena will likely illuminate the fundamental properties of this mechanism in fungi and, potentially, in other eukaryotic organisms.

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Fungal Interactions with Plants: Impact on Agriculture and the Biosphere

VI

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Plant Pathogenic Fungi

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INTRODUCTION

Fungi have developed a plethora of strategies to colonize plants, and these interactions result in a broad spectrum of outcomes ranging from beneficial interactions to death of the host. With respect to plant pathogens, fungi represent probably the most diverse group of ecologically and economically relevant threats. Fungal plant pathogen species are primarily in the phyla Ascomycota and Basidiomycota. Among ascomycetes, plant pathogens are in various classes such as the Dothideomycetes (e.g., *Cladosporium* spp.), Sordariomycetes (e.g., *Magnaporthe* spp.), or the Leotiomycetes (e.g., *Botrytis* spp.). Basidiomycetes are represented by the two largest plant pathogen groups: the rusts (Pucciniomycetes) and the smuts (spread among the subphylum of Ustilaginomycotina).

Fungal infections cause an enormous spectrum of disease symptoms. Traditionally, fungal plant pathogens are divided into two main groups: biotrophic pathogens, which form intimate interactions with plants and can persist in and utilize living tissues (biotrophs), and necrotrophic pathogens, which kill the tissue to extract nutrients (necrotrophs). In addition to these two groups, members of another group, hemibiotrophic pathogens, start as biotrophs and then switch to become necrotrophs. Necrotrophs cause necrosis and eventually even death of infected plants. Symptoms caused by biotrophs in many cases may appear mild. Nevertheless, some of the economically most devastating pathogens belong to this group. Fascinating interactions can be found in highly specified biotrophic interactions, where organ development of the host is massively modulated by the microbe, e.g., leading to formation of pseudo-flowers (rust) or plant tumors (smuts), to promote fungal propagation. In this article, we give an overview of the biology of plant pathogenic fungi with a focus on differentiation of pathogenic structures and the function of virulence factors. The impact of plant pathogenic fungi on agriculture is discussed, and important model organisms are profiled.

IMPACT OF FUNGAL PATHOGENS ON AGRICULTURE

Phytopathogenic fungi have been devastating threats throughout the history of agriculture. In ancient times, plant diseases were blamed on gods, which was a major driving force for the development of religious beliefs. In Greece, Demeter was deified as the god of grain and fertility from about 1300 BC. In the ancient Roman empire from the 5th century BC, Ceres (*crescere* = growing) appeared as Demeter's female pendant. Ancient Roman religion held a spring festival (Robigalia) for the god Robigus, who was the personal manifestation of agricultural disease. Namely, this disease (Robigo) is a form of wheat rust causing red color, which was mythologically linked to the god Mars, a god of both agriculture and bloodshed. To please Robigus and prevent crops from being destroyed by rust disease, the Romans sacrificed red dogs, foxes, and cows—with probably uncertain success.

The first relevant scientific contribution to the knowledge of fungal plant disease can be attributed to Theophrastus (370–288 BC), a student of Aristotle. In his botanical studies, he described the appearance of rust diseases on different host species and in the context of landscape. In retrospect, it appears staggering that for almost 2,000 years there was hardly any scientific progress on the understanding of plant infections, which were continually interpreted as the wrath of God. Besides smut fungi infecting monocot crops such as barley (e.g., *Ustilago hordei*), wheat (e.g., *Ustilago tritici*), or maize (e.g., *Ustilago maydis*), rusts are traditionally serious threats in agriculture. In the 17th cen-

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tury, wheat stem rust (*Puccinia graminis* f. sp. *tritici*) was found to correlate with the presence of barberry (*Berberis vulgaris*) plants growing near wheat fields. Consequently, these plants were eliminated to control rust infection of wheat. This rather simple cure was successful, since the *P. graminis* sexual life cycle requires barberry as an intermediate host that is infected by monokaryotic basidiospores to form spermatia (1). A fungal plant pathogen with enormous agricultural and cultural influence throughout human history is *Claviceps purpurea*, the causal agent of ergot of rye, barley, oats, and wheat. Ergot sclerotia, which contain a broad spectrum of toxic alkaloids including LSD, replace kernels in the heads of crops and thereby cause contamination of harvested grains and, finally, flour. Numerous epidemics of the resulting human diseases, such as "devil's curse" or "holy fire," have been reported, with many thousands of victims and social and political consequences (1).

Fungal epidemics also have had significant social and economical impact. One historical example is the coffee rust, *Hemileia vastatrix*. Until the 1870s, Ceylon (Sri Lanka) was one of the world's greatest coffee producers. This dramatically changed after *H. vastatrix* reached Ceylon in 1875. From 1870 to 1885, its coffee production dropped about 95%, and the fungus completely destroyed coffee plantations in this region, which today is of course mainly known for its tea. Today, coffee rust is still a significant threat to coffee production, with recent outbreaks in Central and South American growth regions (2).

While in modern agriculture, most fungal pathogens can principally be controlled by modern crop management, massive epidemics with devastating yield losses still occur. Recent examples of this are epidemics caused by the Asian soybean rust (*Phakopsora pachyrhizi*) and wheat blast outbreaks in several Asian countries caused by Brazilian *Magnaporthe oryzae* isolates (3). In the following section, we give an overview of a (noncomprehensive) selection of plant pathogenic fungi that play important roles in agriculture and plant pathology research.

FUNGAL MODEL SYSTEMS IN AGRICULTURE AND RESEARCH

Botrytis and *Sclerotinia* spp.

Botrytis cinerea and *Sclerotinia sclerotiorum* are the best examples of broad-host-range, necrotrophic plant pathogens. They were originally treated as types of *Sclerotinia*, and largely on the authority of De Bary, *B. cinerea* had been considered the conidial form of *Sclerotinia fuckeliana* (4). According to current phylogeny, *B. cinerea* belongs to the genus *Botrytis,* and its sexual form is *Botryotinia whetzel*, family Sclerotiniaceae (5). The genus *Botrytis* consists of 22 species and includes pathogens of monocotyledons and dicotyledonous plant species (6). Except for *B. cinerea*, all other species are considered specialists with a narrow host range, with the exception of *Botrytis fabae*, which can infect species of the genera *Vicia*, *Lens*, *Pisum*, and *Phaseolus* (6). Whereas most *Botrytis* spp. have a narrow host range and the majority of them infect monocotyledonous plants, *B. cinerea* belongs to a distinct phylogenetic clade of broad-host-range species which infect predominantly dicotyledonous plants. Other members of this clade are *Botrytis pelargonii*, *Botrytis fabae*, *Botrytis pseudocinerea*, and *Botrytis calthae* (5, 7). Based on a multiloci analysis, *B. cinerea* morphological species were further divided into two genetic groups, group I (referred to as *Botrytis pseudocinerea*) and group II (*B. cinerea sensu stricto*) (8). Strains in these two groups exhibit differences in their ecology and their resistance pattern to fungicides and are unable to cross with each other, suggesting that they represent phylogenetic species. Recently, a novel group, called *Botrytis* group S, was described (9). This group, which is closely related to but distinct from *B. cinerea* and the more host-specific species *B. fabae*, is widespread in strawberry fields in Germany but mostly absent in other regions. Among the various species of *Botrytis, B. cinerea* strains B05.10 and T-4 have been used in most studies. Both strains belong to group II and can be considered model strains in studies to elucidate *B. cinerea* pathogenicity.

The most familiar disease caused by *B. cinerea* is gray mold. This disease is widespread and results in serious economic damage worldwide. It is most destructive on mature or senescent tissues of dicotyledonous hosts. Usually the fungus gains entry to such tissues at an early stage of crop development and remains quiescent until the environment is conducive and the host physiology changes. Therefore, serious damage is caused in storage following harvest of apparently healthy crops. *B. cinerea* also causes massive losses in field- and greenhouse-grown horticultural crops prior to harvest, primarily in fruits, vegetables, and ornamental flowers.

The most common route of *B. cinerea* infection is by conidia, which penetrate the cuticle with the aid of nonmelanized appressoria. When plants are inoculated with mycelium, a different form of penetration takes place, whereby hyphae growing on the plant surface heavily ramify into short bulbous cell aggregates that

resemble the "infection cushions" or "complex appressoria" of the closely related fungus *S. sclerotiorum*. At the end of the infection cycle, the fungus develops sclerotia within dying host tissues, which represent an important survival mechanism. Mycelium can also survive within infected dead host tissues and inside some seeds to serve as primary inoculum. *B. cinerea* also forms microconidia from phialides abundantly in aging cultures, which function primarily as spermatia. The sexual cycle involves the spermatization of sclerotia, leading to the production of apothecia and asci with eight binucleate ascospores.

Cladosporium fulvum (*Passalora fulva*)

C. fulvum (*P. fulva*) is a nonobligate biotrophic fungal pathogen that is a causal agent of tomato leaf mold (10, 11). Until now the teleomorph stage of *C. fulvum* has never been observed in nature or in laboratory conditions. The infectious cycle starts with germination of conidia on the lower side of the tomato leaf. Subsequently, the runner hypha enters the host leaf only through the open stomata without differentiation of an appressorium and exclusively colonizes in the extracellular space of tomato leaves. At 12 days postinfection *C. fulvum* produces many conidiophores that block the stomata and result in some chlorosis or necrosis on the leaf (11). *C. fulvum* was a serious disease of tomato that caused big losses until the 1960s; however, since the introduction of different *Cf* resistance genes into commercial tomato cultivars, this mold disease has been kept under control very efficiently. Although *C. fulvum* is no longer a threat for tomato production, it is a valuable model for the study of plant-fungus interactions.

C. fulvum belongs to the Dothidiomycete class, which contains more than 6,000 species that can infect all types of crop plants. Therefore, the information obtained from the *C. fulvum*-tomato pathosystem can be used to enlighten our understanding of other plant-microbe interactions. For example, homology-based BLAST search revealed the presence of two functional homologs of chitin-binding proteins of *C. fulvum*, Avr4 and Ecp6, in various phytopathogenic fungal species, including *Mycosphaerella fijiensis* (banana pathogen), *Dothistroma septosporum* (pine tree pathogen), *Zymoseptoria tritici* (wheat pathogen), and *M. oryzae* (rice pathogen) (12–16). Like Avr4 from *C. fulvum*, Avr4 from *M. fijiensis* can also bind chitin and protect the fungus from plant chitinases (14). Furthermore, Avr4 from both *M. fijiensis* and *D. septosporum* is recognized by tomato Cf-4 resistance protein (13, 14), indicating that this *R*-mediated resistance can be used against these two devastating pathogens of banana and pine, respectively. Functional characterization of LysM-containing proteins from *Z. tritici* (Mg3LysM) and *M. oryzae* (Slp1) showed that, like Ecp6 protein from *C. fulvum*, these proteins also have the ability to scavenge chitin fragments and prevent chitin-triggered immunity (12, 15, 16). The wide distribution of LysM proteins in the fungal kingdom indicates that scavenging of chitin fragments is a general strategy that is used by many pathogenic fungi to prevent induction of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) or microbe-associated molecular pattern-triggered immunity.

M. oryzae

The hemibiotrophic ascomycete *M. oryzae* is the causal agent of rice blast disease. This disease is one of the most devastating diseases of cultivated rice throughout the world, and the annual yield loss due to rice blast disease can be up to 30% of total rice production (17–19). *M. oryzae* can also infect wheat and other small grains; this seriously threatens global wheat production and food security (20). Because of its economic significance and easy genetic and molecular manipulation, *M. oryzae* has become a model system and is widely used for analyzing plant-microbe interactions (21, 22).

M. oryzae is a typical hemibiotrophic pathogen that derives nutrition from living cells at the early infection stage, transits into the necrotrophic stage to kill host cells for nutrition, and then produces asexual spores at later stages. To colonize and infect the rice leaves successfully, the *M. oryzae* conidia develop melanized appressorium, a special infection structure by which they penetrate through the rice cell wall via enormous turgor pressure (23, 24). Once it has successfully penetrated to the plant cell via the penetration peg, the primary hyphae spread through plant cells and develop into bulbous invasive hyphae that are sealed inside the plant membrane structure (25). Associated with the invasive hyphae, *M. oryzae* also develops a biotrophic interfacial complex structure, which is significant for the accumulation and secretion of effectors to avoid or suppress plant defense responses during the biotrophic phase (26). Subsequently, the primary hyphae of *M. oryzae* invade adjacent cells via plasmodesmata and switch to the necrotrophic stage, forming typical necrotic lesions on the surface of the rice leaves (22, 23). A number of studies have demonstrated that the cAMP signaling pathway is involved in surface recognition and appressorium initiation at the early infection stage and that the Pmk1 MAP kinase (MAPK) pathway regulates appressorium formation, appressorium penetration, and invasive growth after penetration, which

are two other critical infection processes in the infection cycle of *M. oryzae* (27).

For successful infection, *M. oryzae* suppresses the host immune response manifested as an oxidative burst or plant cell death during the biotrophic stage. This suppression is achieved by effectors, which are pathogen molecules that have an impact on the outcome of the interaction with the host; they are discussed extensively in the section "Virulence Tools of Plant Pathogenic Fungi." Other effectors are associated with the necrotrophic stage. A secreted effector protein, Slp1, of *M. oryzae* specifically accumulates at the interface of fungus-rice cells at the early stages of infection and competes with CEBiP (a chitin-binding protein of rice) for chitin oligosaccharides binding through LysM domains, thus interfering with chitin-triggered immunity in the host (16). *M. oryzae* can also secrete various effectors to suppress BAX (Bcl-2-associated X protein)- and/or Nep-1 (Necrosis- and ethylene-inducing peptide-1)-mediated programmed cell death (PCD) (28, 29). The cytoplasmic effector AvrPiz-t of *M. oryzae* can inhibit the activity of rice RING E3 ubiquitin ligase APIP6, thus suppressing the oxidative burst and PTI in rice and contributing to virulence (30). The secretory protein MSP1 can induce autophagy cell death, H_2O_2 production, and defense responses in rice leaves and contributes to pathogenicity of *M. oryzae* on barley leaves (31).

U. maydis

U. maydis belongs to the group of smut fungi, which comprises more than 1,500 species parasitizing a wide range of mono- and dicotyledonous plants. Although of rather moderate economic relevance, *U. maydis* has been developed as the prime model organism for smut fungi and also is one of the best-characterized biotrophic plant pathogens in general (32). Accessibility to reverse genetics, simple axenic growth, and a compact haploid genome with little redundancy make *U. maydis* an excellent tool for molecular fungal genetics (33). Its biphasic life cycle consists of a nonpathogenic, saprophytic phase and an infectious stage, which is initiated upon mating of compatible cells on the plant surface. *U. maydis* infects primordia of all aerial parts of the maize plant and establishes a biotrophic interaction, where the fungus spreads both inter- and intracellularly. Massive proliferation of fungal hyphae coincides with the formation of plant tumors, which represent the most prominent symptoms of the corn smut disease caused by *U. maydis* (34). In recent years, intensive efforts have been invested in the identification and functional characterization of *U. maydis* virulence factors, mainly secreted effector proteins that act in the host tissue to support fungal virulence and reprogram host tissue, promoting tumor formation (35). Details on the functional characterization of *U. maydis* pathogenic development and its virulence factors are presented later in this article.

Z. tritici (*Mycosphaerella graminicola*)

The ascomycete *Z. tritici* (syn. *M. graminicola*; the teleomorph of *Septoria tritici*) is a hemibiotrophic (or necrotrophic) fungal pathogen that is a causal agent of *S. tritici* blotch (STB) of wheat (*Triticum aestivum*) (36–38). Like *C. fulvum*, *Z. tritici* is a dothidiomycete. Both asexual and sexual stages of *Z. tritici* have been reported in all STB-occurring areas (39). *Z. tritici* causes one of the most economically important foliar diseases of wheat and is responsible for up to 30 to 50% of annual yield losses (40). STB disease control is mainly based on fungicide treatment; however, the fungus has developed resistance to multiple fungicides (41, 42) and it is now difficult to control this disease worldwide. Currently, only a few wheat cultivars that have resistance trait(s) to STB are used worldwide to control this disease. Despite its agricultural importance, very few genes are known to be involved in the virulence and pathogenicity of *Z. tritici*. Moreover, most of these known virulence factors belong to global regulators and cell signaling gene families such as MAPK pathways and cyclic nucleotide signaling (43–45).

KEY STEPS OF FUNGAL PATHOGENESIS

Fungal Host Attachment and Penetration

To find their hosts, fungal spores are dispersed by wind, water, or insect vectors. These spores are able to attach to various surfaces including host or nonhost plants. Such attachment prevents spores from being washed away from the host surface before penetration. Secretion of adhesive extracellular matrix from spores is essential for firm adhesion to the host surface. Hamer et al. showed that the attachment of *M. oryzae* spores to various surfaces requires spore-tip mucilage, which is stored in a periplasmic compartment at the conidial apex and is released upon hydration of conidia that results in rupturing of the spore wall (46).

In favorable conditions, the infection cycle of plant pathogenic fungi starts with spore germination and filamentous germ tube formation, which require complete cell reprogramming and specific regulatory networks. Upon spore germination, runner hyphae show polarized cell growth and grow along the host plant surface, which depends on recognition of distinct physical (sur-

face hardness, hydrophobicity) and chemical (cutin monomers, leaf waxes) stimuli (22). How fungi perceive these signals is not clear. A G-protein-coupled receptor, Pth11, and cognate G-α- and G-βγ-subunit proteins are essential for appressorium development on hydrophobic surfaces (19, 47).

Mechanisms of Host Penetration

Many phytopathogenic fungi, such as *M. oryzae*, *Colletotrichum* spp., and *Alternaria* spp., differentiate a dome-shaped appressorium cell to penetrate their hosts by directly breaching the cuticle layer (48, 49). The dome-shaped appressorium cell has a specialized cell wall enriched with chitin and an inner layer lined with melanin. The formation of a melanin layer is essential to *M. oryzae* appressoria, since it allows the generation of high osmotic pressure by the accumulation of glycerol (19, 23, 50). The appressorium tightly attaches to the host surface and applies a turgor-driven mechanical force onto a thin penetration peg that ruptures the cuticle at the base of the appressorium and grows into the underlying plant epidermal cell. Appressorial turgor pressure can be up to 8.0 MPa (50). This physical pressure and some degree of enzymatic degradation of the host cuticle and cell wall enable the peg to penetrate host tissues.

Recent studies, mainly on *M. oryzae*, have provided impressive insights into the regulation of signaling cascades and cellular reorganization during the formation of appressoria and their function to redirect fungal growth and initiate host infection (51). Saunders et al. (248) have reported that initiation of appressorium development in *M. oryzae* is regulated by a DNA replication-dependent checkpoint (Never-in-Mitosis 1). After a fungal spore attaches to the host leaf surface, the germ tube enters into a single round of mitosis, which is required for the cellular differentiation of appressoria (51). In addition, it has been recently shown that actin remodeling is required for appressorium development. *M. oryzae* appressoria invade plants via a septin-dependent mechanism (24). Septins form a hetero-oligomeric ring at the appressorium pore to organize a toroidal F-actin network and link this network to the appressorium membrane. Formation of the septin ring also serves as a diffusion barrier to prevent the diffusion of proteins that are involved in membrane curvature and F-actin polymerization. In this way, septins provide the cortical rigidity and membrane curvature for the extension of the penetration peg during host cuticle breach (24). Moreover, septins mediate assembly of the exocyst complex at the appressorium pore, which is necessary for appressorium repolarization (52).

Other pathogens differentiate a hyphal tip swelling of the infection hypha, which reflects the point of host penetration, but this structure is not separated by a cell wall, and its cell wall is not fortified by melanization. Although these structures are often termed appressoria, it is likely that the activity of lytic enzymes rather than a mechanical force facilitates epidermal penetration from this type of infection structure. Pathogens that form nonmelanized, nonseparated appressorial swellings include *B. cinerea* and *U. maydis* (53–55). A third group of species, including *C. fulvum* and most rust fungi, penetrate their hosts through a natural opening (stomata) of the host plants (11, 56).

Fungi that use stomata for penetration of the host have evolved directional growth patterns on the host leaf surface to locate and recognize stomatal guard cells. Physical contact of runner hyphae with topographical signals of the host leaf surface controls both oriented growth and appressorium formation. This movement of the fungal germ tubes in response to topography of the leaf surface toward the host stomata is called thigmotropism (57). Hoch and colleagues showed that the differentiation signal for *Uromyces appendiculatus* is simply the height of the ridge present on host leaf surface. Upon contact with the stomatal ridge (optimum ridge height is 0.5 m) of the leaf, the germ tube of the rust fungus *U. appendiculatus* undergoes thigmo-differentiation and forms an appressorium directly over the stomata, and the penetration peg enters the host (57). Subsequently, the pegs differentiate into invasive hyphae that rapidly colonize to epidermal and mesophyll tissues.

LIFESTYLES OF PLANT PATHOGENIC FUNGI

Plant pathogenic fungi are collectively those species that derive nutrients from plants and have a negative effect on plants' health. Similar to the situation in the entire kingdom of Fungi, they vary in nutritional requirements; certain types of pathogens completely depend on their host (these are called obligate parasites); others form a close association with the host but may also complete the life cycle of the plant. Such facultative pathogens can prosper in a variety of environments other than the organism(s) in which they cause disease.

The traditional categorization of plant pathogens (necrotrophs, biotrophs, hemibiotrophs) differentiates fungi according to their pathogenic lifestyle and the way they feed on the host. Recent findings suggest that the division is less strict than previously assumed; however, this categorization defines basic denominators

that are common to all organisms in each class and simplifies the discussion of pathogenic lifestyles.

Biotrophs

Pathogens with a biotrophic lifestyle are either obligate or nonobligate. Obligate biotrophs are the causative agents of powdery mildew (Ascomycota) and rust (Basidiomycota) diseases. Downy mildews and white rusts are also obligate parasites; however, these species are Oomycota (protozoa) and will not be discussed here. We will also restrict the discussion to parasites and therefore will not include nonpathogenic biotrophic fungi such as mycorrhizae (58).

The obligate biotrophic pathogens, rusts and powdery mildews, have evolved to match the life cycle of their host plants and depend on their host for completion of their life cycle. Rusts in particular have developed different spore types and developmental patterns, including infection of an alternative host upon senescence of the primary host (59). Rust diseases are caused by filamentous, basidiomycete fungi in the order Pucciniales (60). Eurediniospores (dikaryotic conidia) germinate on a leaf of the primary host plant and develop invading hyphae that grow into the mesophyll space through stomata. The invading hypha differentiates into substomatal vesicles, which produce primary hyphae. Powdery mildew diseases are caused by haploid filamentous ascomycetes in the order Erysiphales. Conidia of these fungi germinate on the leaf surface and penetrate the epidermal cells with the aid of appressoria (61, 62). Following transition from the surface to an internal space, these fungi cross the wall of mesophyll (rusts) or epidermis (powdery mildew) cells and differentiate haustoria, which are unique infection structures that serve as mediators of nutrient acquisition. The haustoria form behind the plant cell wall, but they do not disrupt the cell membrane. Instead, they push and invaginate the cell membrane, establishing a sustainable "cell within cell" complex. This fungus-plant hybrid structure consists of the plant cell cytoplasm and tonoplast, a polysaccharide-rich extra-haustorial matrix, and the haustorial wall and membrane (63). Following establishment within the host cells, the haustoria deploy a range of transporters to drive nutrients from the infected host cells into the haustorium (64–66). At the same time they also secrete effectors that suppress the host defense and keep the invaded cells alive (67–70) (see the section "Virulence Tools of Plant Pathogenic Fungi").

It is assumed that obligate biotrophic species are limited in their ability to utilize common substrates as energy sources, and therefore they fully depend on their host as an energy source, which makes them obligatory parasites (58, 71). Genomic analyses indicate a loss of genes in key metabolic pathways, such as genes in the nitrogen and sulfur assimilation pathways (60, 61, 72, 73). It was also found that the genomes of the powdery mildew *Blumeria graminis* (61), the rusts *P. graminis* and *Melampsora larici-populina* (60), and the nonobligate biotroph *U. maydis* (33) all have reduced numbers of genes encoding hydrolytic carbohydrate active enzymes (74). All these findings support the loss of some biosynthetic activities in obligate biotrophs. It is important to note, however, that there is no clear evidence that the loss of biosynthetic and metabolic capacity is the limiting factor in the ability of obligate biotrophic fungal plant pathogens to grow out of their host (75, 76). Furthermore, although obligate biotrophic species rely on their host plants for completion of their life cycle, all of them can be grown, although for a limited time, in axenic cultures (77, 78), suggesting that they have at least some capacity to extract nutrients and energy from a nonliving environment. An alternative hypothesis is that in obligate biotrophs the regulation of metabolic gene expression is dependent on signals and signal gradients that are only found in host plants. According to this hypothesis, obligatory biotrophs utilize cues from the host to control their metabolic capacity. Evidence that supports this hypothesis is the coordinated regulation of expression of metabolic genes in barley powdery mildew, during both development and pathogenic attack (75), and the expression of important metabolic and uptake functions in rust haustoria (66, 79, 80).

Nonobligate biotrophic plant pathogens are taxonomically more variable than obligate biotrophs and can be found in a wider range of genera. Among them are the smuts (Basidiomycota, *Ustilaginales*) and certain species of *Claviceps* (Ascomycota, *Claviceptacea*). Unlike the obligate species, nonobligate species can survive and live without the host. For example, the ergot disease fungus *C. purpurea* behaves as a true biotroph *in planta*, but it can be easily grown in axenic culture (81). Infection is initiated by wind-borne ascospores that germinate on the pistil surfaces of grass florets at anthesis. Following penetration through stigmatic hairs, the fungus reaches and colonizes the ovarian tissue, grows down toward the base of the ovary, and establishes a specific and persisting host-pathogen interface. A mycelial stroma develops in the ovary and produces masses of conidiospores that are exuded into a sugar-rich fluid derived from phloem sap. About 2 weeks postinfection, sclerotia are formed, which serve as overwintering structures. *C. purpurea* shows no

necrotrophic growth pattern in any phase of infection, and it does not kill host cells in advance of colonization. Although it does not produce classical haustoria, intracellular hyphae, which are completely covered by the host plasma membrane, could have haustorial functions (82). Among smut fungi, the maize pathogen *U. maydis* stands out because of its amenability to molecular genetic manipulation and its small genome size. *U. maydis* enters primordia of all aerial organs of the maize plant, where it locally induces formation of tumors, in which fungal spores develop (32).

Necrotrophs

As opposed to biotrophic pathogens, which feed on living plant tissues, necrotrophic plant pathogens feed on dead tissue. It is important to make a distinction between true necrotrophic pathogens, which attack and kill healthy plants, and secondary necrotrophic-like pathogens, which are saprophytic in nature but may occasionally infect plants that have been previously weakened, e.g., by other pathogens, injury, or abiotic effects. Here we only discuss true necrotrophs.

The basic concept of necrotrophy is defined as the mode of infection in which the pathogen kills the tissue before colonization (83). This definition is somewhat oxymoronic since at least the initial contact of the pathogen is with a living tissue. Indeed, the early stages following the first encounter with the host are most critical; to survive, the pathogen must subvert the plant defense and generate a zone of dead (necrotic) tissue in which it will be protected from the host and on which it can feed. The following stages of necrotrophic diseases are characterized by spreading of necrosis around the initial infection zone, which precedes pathogen progression. This two-phase strategy entails a "survive or die" scenario at the initial stage, in which the pathogens are in direct confrontation with the host defense, and a more quantitative scenario during disease spread. As described, biotrophic interactions also have initial and late stages; however, in this case the early stage does not involve direct conflict with the host defense, but rather a silent "sneaking in" strategy, while all later stages necessitate close contact with the living host and hence a continuous conflict with the host defense. Accordingly, biotrophic pathogens also have a "survive or die" stage at the first encounter with the plant defense, but only after penetration into the tissue, prior or during establishment of the biotrophic interface. Because biotrophic pathogens thrive to keep the tissue alive, the plant defense strategy at this critical stage is a suicidal one in the form of a hypersensitive response (HR), while the pathogen strategy is prevention of this response.

The realization that necrotrophic pathogens must cope with the plant defense during the first encounter and overcome it in order to initiate infection highlights the true nature of this type of pathogen. It is now clear that necrotrophic pathogens use an array of specific molecules (effectors) and mechanisms to cope with and manipulate the host defense during infection, in a manner that is not less sophisticated than biotrophic pathogens. This is in contrast to an earlier paradigm that assumed a more simplistic strategy of massive killing of tissue using a battery of hydrolytic enzymes and toxins (8). Compared to true biotrophic pathogens, true necrotrophic pathogens include a much wider range of species that belong to diverse genera. Although the two-phase paradigm holds true for all of the necrotrophic pathogens, not all necrotrophs are made equal. It is convenient to divide all necrotrophic pathogens to narrow-host-range and broad-host-range species (84). Host specificity of necrotrophic species is conferred through the production of host-specific toxins (HST), which are essential pathogenicity factors on a compatible host. Examples include the HSTs from *Cochliobolus* spp.: HC-toxin from *Cochliobolus carbonum*, T-toxin from *Cochliobolus heterostrophus*, and victorin from *Cochliobolus victoriae* (85). Other examples of HSTs are ToxA toxins, which determine the host range of the wheat tan spot, and blotch diseases caused by *Pyrenophora tritici-repentis* and *Parastagonospora nodorum*, respectively (86). In all cases in which HSTs are involved, they interact with a specific host gene, much in the same way that Avr or effector proteins interact with resistance proteins (R proteins). In fact, HSTs share many characteristics with avirulence gene products: they have a primary virulence function, they are specifically recognized by host resistance counterparts, and they can be recognized by the plant immune system as virulence factors. It therefore seems appropriate to define HSTs as effectors. This is discussed in more detail in reference 249.

Broad-host-range necrotrophs lack HSTs and may attack a wide range of plant species. This group is best represented by *B. cinerea* and the taxonomically closely related *S. sclerotiorum*. Both fungi have considerably broader host ranges than most plant pathogens (200 to 400 plant species), and each causes multimillions of dollars in pre- and postharvest annual crop losses worldwide (87–89). Melanized sclerotia play a central role in the life cycle of both fungi by germinating either vegetatively for local colonization or carpogenically to initiate the sexual cycle, including production of apothecia from which ascospores are released. There are a plethora of additional broad-host-range necrotrophs,

including many species in the genus *Alternaria* and others in different taxa. While simply inherited resistance traits are sufficient for conferring protection against host-specific necrotrophs, the genetic basis of resistance against broad-host-range necrotrophic pathogens is much more complex and typically quantitative (83, 84). This is in contrast to R-gene-mediated resistance or HST-blocking genes, which provide complete resistance. For this reason, it is much harder to control broad-host-range necrotrophic pathogens, which might partially explain their growing economical importance.

Regardless of host range, the basic strategy of all necrotrophic plant pathogens is to convert living tissue into dead material. To achieve this goal, necrotrophs target the host cell death pathways. Studies in recent years show that induced host cell death accompanies all stages of necrotrophic infection, and different types of cell death pathways are targeted. At early stages of infection, necrotrophs produce and secrete necrosis-inducing effectors such as Neps, cerato-platanin, and certain glycosyl hydrolases to induce local necrotic cell death (83, 84, 90–92). At later stages, they probably require different types of effectors, which induce PCD. For example, *S. sclerotiorum* produces oxalic acid, which induces PCD in the host while suppressing autophagy (93, 94). It is important to note that the role of autophagy in pathogenicity is still vague: in some cases it is probably a salvation mechanism that alleviates damage and rescues host cells following PCD, but in other situations it serves as a prodeath mechanism (95). Production of reactive oxygen species (ROS) is another central element that mediates the necrotrophic lifestyle (96). Similar to autophagy, ROS probably have multiple effects, and therefore, although they are considered a defense response, they might also serve as a virulence factor, at least in necrotrophic interactions. For example, active modulation of the host redox status appears to be crucial for *B. cinerea* and *S. sclerotiorum* diseases (8, 91, 94, 97). In fact, *B. cinerea* seems to exploit the plant's oxidative burst and might even contribute to ROS production. Similar results have been obtained for other necrotrophic fungi, e.g., in *Leptosphaeria maculans*, a pathogen of *Brassica napus* (98).

Originally, necrotrophy was interpreted as rather brutal toxin-assisted maceration of the host tissue. Indeed, studies showing the use of hydrolytic enzymes by necrotrophic pathogens go back as far as the beginning of the previous century (4, 99). While current studies show that these enzymes do not work alone, and they might have additional roles other than sheer hydrolysis of plant polymers, it is important to remember that successful necrotrophs rely on massive secretion of such hydrolytic enzymes for maceration and utilization of the plant tissues as a nutritional source. The relatively large number of genes coding for cell-wall-degrading and other hydrolytic enzymes that are present in genomes of necrotrophic fungi support this notion (88, 100). The high redundancy of many of these enzymes, which is unusual in fungi, also supports their essentiality in these species. Finally, transcriptome and secretome analyses show that a large number of these enzymes are produced and secreted at various stages of the infection process (92, 101, 102).

Hemibiotrophs

Intuitively, hemibiotrophic pathogens are those species that combine biotrophic and necrotrophic lifestyles. Most scientists define hemibiotrophs as species that have a variable length of a biotrophic phase before switching to necrotrophy (103–105). This definition entails an initial true biotrophic phase that is mediated by special biotrophic organs. During this phase fungal pathogens also secrete effectors to suppress the plant defense. At the end of the transient biotrophic stage, the fungus undergoes a massive developmental change that mediates the transition from a biotrophic mode to a necrotrophic mode. Two cases best fit this scenario: the rice blast fungus *M. oryzae* and species within the genus *Colletotrichum* (103). Pathogens belonging to these groups answer the classical definition of hemibiotrophy: following penetration into the subepidermal or epidermal cells, they develop specialized hyphae that form close contacts with the host and invaginate the host cell membrane, thereby forming a true (although transient) biotrophic interphase. After a certain period of time that may last from 1 to several days (depending on the species), the fungus switches to a classical necrotrophic mode. This transition includes differentiation of the new type of hyphae, secretion of enzymes and toxins, and delivery of specialized effectors.

Magnaporthe and *Colletotrichum* species are important plant pathogens; they can be cultured and are amenable to genetic manipulations, and they exhibit classical hemibiotrophic behavior. For these reasons, *M. oryzae* and *Colletotrichum* spp. have been used as models for the hemibiotrophic lifestyle. Many other species that belong to a wide range of genera are also considered hemibiotrophic. Among them are important plant pathogens such as *Fusarium* (106, 107), *Verticillium* (108), *Mycosphaerella* (109, 110), and many others. According to a loose definition, all of the species that are not defined as necrotrophs or biotrophs are potentially hemibiotrophs. We believe that this grouping might cause confusion, since most of these

other species do not exhibit a true hemobiotrophic lifestyle. In fact, the common denominator of all of these species is a symptomless stage that varies in length, but in most cases they do not differentiate the typical biotrophic specialized organs and do not form close contact with the host cell, but rather remain in the apoplastic or intracellular sphere. As such, the pathogenic lifestyle of these species includes symptomless, quiescent, latent, or endophytic stages, but they do not meet the criteria of hemibiotrophy as defined above and therefore should not be treated in the same way. For this reason, we limit the discussion of the hemibiotrophic lifestyle to *M. oryzae* and *Colletotrichum* spp. Further, although certain species, including *M. oryzae* and *Colletotricum graminicola*, can cause systemic infections, the main and prevalent mode of *M. oryzae* and *Colletotricum* spp. is foliar and stem infection, and only this mode will be reviewed here.

Hemibiotrophic species seem to have evolved highly successful infection strategies, and they are among the most aggressive plant pathogens. Concerning host range, *M. oryzae* can cause disease in a limited number of grasses; however, it is most well known for the rice blast disease, the most destructive disease of rice (17, 19, 103). *Colletotrichum* spp. cause anthracnose diseases in more than 600 dicot and monocot plant species and include severe pathogens of important crops (48). Examples are *C. graminicola*, which causes anthracnose in maize (111), *Colletotrichum orbiculare*, a pathogen of cucurbits (112), *Colletotrichum acutatum*, a pathogen of strawberries and tomatoes (113), *Colletotrichum lindemuthianum*, which attacks bean plants (105), and *Colletotrichum gloeosporioides*, which can attack a wide range of plant species (114). While some species have a relatively wide host range, others are highly specific and specialize on a single host, and therefore they are defined as *forma speciales* (f. sp.). An example is *C. gloeosporioides* f. sp. *aeschynomene*, which specifically attacks the legume weed *Aeschynomene virginica*. Its high aggressiveness and restricted host range were exploited to develop a bioherbicide product (Collego) from conidia of this fungus (115).

Infection of *M. oryzae* and *Colletotrichum* spp. is typically initiated by conidia. Following sensing of the leaf surface, conidia germinate and form a short germ tube. Interestingly, germination of conidia on a leaf or on surfaces with similar chemophysical properties differs significantly from germination in carbohydrate-rich media. When *C. gloeosporioides* is pregerminated in a rich medium, conidia are unable to infect plants. Hence, pathogenic and nonpathogenic germination styles were identified (116, 117). Studies with *M. oryzae* and *C. gloeosporioides* also have revealed that cellular processes during pathogenic germination are well orchestrated, involving cell cycle and cell architecture as well as PCD (118). Pathogenic germination ends with the formation of a well-developed, dome-shaped appressorium, which has become the hallmark of hemibiotrophic species, in particular *M. oryzae*. A series of groundbreaking studies on appressorium formation in *M. oryzae* has elucidated the structural, mechanistic, and molecular mode of action of appressoria (22, 49, 119, 120). Clearly, the main function of appressorium in these species is bringing the fungus underneath the cuticle without alarming the plant defense. This is achieved by poking an extremely thin hole in the cuticle by physical force, thereby avoiding release of plant-derived elicitors. Once the fungus has successfully moved from the surface of the leaf to the subcuticular space, a transient biotrophic phase begins. Similar to the situation in the other lifestyles, these late infection stages are less uniform and vary in details from one species to another.

In *M. oryzae*, the penetrating peg breaches the wall of the cell underneath the appressorium and immediately differentiates into a thin filamentous primary hypha that grows in the cell lumen and invaginates the host plasma membrane. In this respect, the first biotrophic stages of *M. oryzae* resemble the biotrophic development that is typical of powdery mildews. The primary invasive hyphae differentiate into bulbous invasive hyphae that become sealed in an extra-invasive hyphal membrane compartment and exhibit pseudohyphal growth as they fill the invaded cell (25, 26, 121). At a specific time, the invasive hyphae move to adjacent cells, probably though plasmodesmata, and repeat the same invagination and proliferation steps, while the hyphae in the original cell switch to a necrotrophic behavior and instantly kill the host cell. Clearly, this sequence of events must be closely coordinated with the host defense. Indeed, *M. oryzae* utilizes a range of stage-specific effectors to affect the host, starting at the stage of appressorium formation and continuing for the entire course of the interaction (103, 122, 123). Furthermore, *M. oryzae* uses distinct pathways for secreting cytoplasmic and apoplastic effectors (124). Secretion of apoplastic effectors occurs through the conventional Golgi-mediated secretion pathway. However, cytoplasmic effectors accumulate in the biotrophic interfacial complex.

In *Colletotrichum* spp. there are two main types of biotrophic stages. The first resembles the biotrophic phases of rice blast and powdery mildews, in which the penetration peg breaches the wall of a subcuticular cell

under the appressorium and differentiates a primary hypha, which invaginates the cell membrane, forming a transient biotrophic stage. This type of interaction is best studied in *Colletotrichum higginsianum*, a pathogen of crucifers including *Arabidopsis thaliana* (104). In *C. higginsianum*, biotrophic infection is confined to the initially infected epidermal cell, and development then switches to necrotrophic infection hyphae, which ramify throughout the host cells. The second type of *Colletotrichum* biotrophy resembles the biotrophic phase of rusts, in which primary hyphae first invade the mesophyll and then form intimate contact with mesophyll cells. This mode of development is best exemplified by the maize pathogen *C. graminicola*. Biotrophic invasion of new cells by *C. graminicola* persists at the advancing colony margin. Necrotrophic hyphae develop later in the center of the colony and advance outward, resulting in the overlapping of biotrophic and necrotrophic phases (125, 125). Similar to *M. oryzae*, the genomes of *Colletotrichum* spp. encode a large number of putative effectors. Expression of many of these putative effectors in *C. higginsianum* is highly orchestrated, and they fall into four distinct expression clusters (48). Gene expression studies also show orchestrated expression of many genes of secondary metabolism, at least several of which are suspected to play a role in infection (104, 127). As might be expected, the genomes of these species are also enriched for genes encoding plant cell wall-degrading enzymes (CWDEs), which are expected to be necessary at the necrotrophic phase (104).

Plant Cell Death in Fungal Interactions

In a broad sense, all fungal pathogens might be divided into killers and nonkillers, regardless of the specific lifestyle (128). According to this point of view, the ultimate goal of killer pathogens is conversion of living tissue into dead organic matter, and therefore they are programmed to efficiently kill host cells. Nonkiller pathogens aim to keep their host alive, and therefore they are programmed to prevent host cells from dying. While the details differ from one pathogen to another, this categorization ultimately relies on the general infection strategy: immediate killing or prevention of death of host cells. As a consequence, cell-death-regulating pathways are expected to play a central role in mediating all types of pathogenic fungal-host interactions. For example, it is intuitive to expect that nonkiller pathogens are armed with effectors that suppress PCD, while killer pathogens have molecules that trigger and maintain PCD. This hypothesis is supported by the opposite effects of antiapoptotic genes on infection by killer and nonkiller pathogens. For example, expression of heterologous (e.g., Bcl2, Bcl-xL) or endogenous (BI-1) antiapoptotic genes protects plants against killer pathogens such as *B. cinerea* and *S. sclerotiorum* (129, 130) while increasing susceptibility to powdery mildew (131, 132).

Host Specificity

Another aspect to consider is a possible connection between lifestyle and host specificity. As described above, host range varies considerably, from single-host-specific species to broad-host-range pathogens that are capable of causing different types of diseases either in the same host or on numerous hosts. Obligate biotrophic species seem to have more restricted host specificity than species with hemibiotrophic or necrotrophic lifestyles. Even nonobligate biotrophic species seem to have the capacity to infect a broad range of hosts, as demonstrated by *C. purpurea*, which is capable of infecting more than 400 plant species (81). Moreover, at the genus level even obligate biotrophic pathogens may have a broad host range. For example, powdery mildews cause diseases in over 10,000 plant species, including monocots and dicots, shrubs and trees (133). Hence, species with a broad host range seem to exist in all types of lifestyles. Additionally, as mentioned earlier, some necrotrophic species are highly specialized, as demonstrated by species in the genus *Cochliobolus* (85). Thus, pathogens with a restricted or broad host range exist in the entire array of lifestyles. It is, however, possible that the host range in each class is limited by different types of elements of the plant defense. For example, the take-all fungus *Gaeumannomyces graminis* f. sp. *tritici* is unable to infect oat because it is blocked by avenacine B, a saponin that accumulates in the oat roots (134, 135). However, a closely related species, *G. graminis* f. sp. *avena* can degrade avenacine B and therefore can infect both oat and wheat. In this case, host range is limited by a single defense molecule that confers resistance to the pathogen, while a single fungal enzyme is sufficient to extend the host range to additional plants. In the case of obligate parasites, it is enough for the plant to initiate the HR response for elimination of the pathogen. It is possible that for this reason host range is restricted by recognition factors (resistance/avirulence proteins) that instantly trigger HR, rather than by enzyme- or metabolite-mediated activities.

VIRULENCE TOOLS OF PLANT PATHOGENIC FUNGI

Disease establishment of fungal pathogens on their host plants relies on diverse virulence factors, including effector proteins, secondary metabolites, and even small

RNA molecules. These virulence factors could be involved in host penetration, prevention, or suppression of host defenses and nutrient acquisition. In addition to these factors, virulence of plant pathogenic fungi also requires morphological transitions to establish host colonization and complete their life cycles. On the other side, plants have evolved diverse innate immune systems to defend against pathogen infection. The pattern recognition receptors localized on the plant cell membrane are designed to recognize PAMPs. These microbial signatures (such as flagellin, chitin, and glucan fragments) trigger the basal resistance response called PTI, including oxidative burst, ion fluxes, activation of MAPK signal pathways, synthesis of plant hormone, and callose deposition (35, 136, 137). Many fungal virulence factors aim to inhibit or avoid PTI and dampen basal defenses, but they can also be perceived by plant leucine-rich repeat proteins, which are known as R proteins, triggering an effector-triggered immunity (138–140). The effector-triggered immunity defense is often accompanied by localized plant cell death or HR (138, 141). To infect plant hosts effectively, these pathogens with different lifestyles adopt diverse strategies and/or virulence factors to manipulate the physiology of host plants and thus to interfere with plant immunity systems and promote virulence or compatibility effectively.

Mycotoxins

Under favorable environmental conditions, fungal pathogens can secrete a variety of secondary metabolites, including mycotoxins and secretory proteinaceous toxins, which induce cell death and contribute to the pathogenicity on plant hosts (142). These toxins can be divided into general toxins and HSTs.

General toxins

The best-known non-host-specific sesquiterpene phytotoxin is botrydial, which is produced by the necrotroph *B. cinerea* during plant infection (143). Botrydial induces chlorosis and collapse of French bean tissues (144) and causes necrosis in *A. thaliana* leaves (145). Further studies demonstrated that botrydial can also induce the accumulation of ROS and phenolic compounds, callose deposition, expression of the defense-associated genes *PR1* and *PDF1.2*, which are involved in salicylic acid (SA) and jasmonic acid signal pathways, respectively, and the expression of HR marker *HSR3* in host plants (145). Additionally, deletion of one of the botrydial biosynthetic pathway genes, *bcbot1*, led to reduced virulence only in the T4 strain, indicating that botrydial is a strain-dependent virulence factor (146).

Oxalic acid (OA), another well-known non-host-specific toxin, is considered an important pathogenicity factor in *S. sclerotiorum*. This pathogen secretes copious amounts of OA to rapidly kill host plants, chelate Ca^{2+}, and reduce pH for activation of CWDEs during infection to facilitate pathogenicity (147). Consequently, OA-deficient mutants were found to be nonpathogenic (148). However, several studies indicated that OA has more significant roles than only functioning as a direct killer, because it can suppress host oxidative burst (149) and trigger ROS-mediated apoptotic-like PCD (94). Recent studies also indicate that OA can reduce the plant's redox environment conditions ahead of advancing hyphae (93, 97), suppressing autophagy and callose deposition during an early infection stage, but once infection is established, promoting host PCD at a later infection stage (93).

Sphinganine mycotoxin fumonisin B1 produced by several *Fusarium* spp. can induce plant cell death, accumulation of camalexin and ROS, callose deposition, and expression of defense genes in *A. thaliana* (150, 151). Fumonisin B1 also contributes to fungal pathogenicity and is necessary for development of disease symptoms on susceptible maize seedlings (152, 153). Some *Fusarium* spp., such as *Fusarium graminearum*, can produce multiple trichothecene mycotoxins, including deoxynivalenol (DON), acetyldeoxynivalenol (3-ADON or 15-ADON), nivalenol (NIV) and acetylnivalenol (4-ANIV), and polyketide zearalenone, in infected plant tissues (154, 155). DON can inhibit protein synthesis in eukaryotic organisms and also acts as a significant virulence factor during infection (156–159). Other toxins, such as fusicoccin from *Fusicoccum amygdali* and AAL toxin from *Alternaria alternata*, can induce both expression of pathogenesis-related genes and cell death in host plants (160).

HSTs

HST proteins are produced by a small number of host-specific necrotrophs. These proteins can function as effectors by interacting with their host targets and utilizing plant resistance signaling pathways to trigger HR and/or PCD for facilitating infection (86, 161). To date, multiple interactions between HST effectors of necrotrophic fungal pathogens and their host target proteins have been reported. Victorin produced by *C. victoriae* can interact with its targets TRX-h5 as well as LOV1 of *A. thaliana*, mimicking the roles of conventional effectors of biotrophic and hemibiotrophic pathogens to activate an HR-like response for promoting disease (162, 163). The *Sorghum bicolor Pc* locus determines the sensitivity to PC toxin protein of the

pathogen *Periconia circinata* (164). This inverse gene-for-gene method also exists in the wheat pathosystem *P. nodorum*. To date, nine pairs of secreted necrotrophic effector-host sensitivity gene interactions have been identified in this system: SnToxA-Tsn1 (165), SnTox1-Snn1 (166), SnTox2-Snn2 (167), SnTox3-Snn3-B1 (168, 169), SnTox3-Snn3-D1 (170), SnTox4-Snn4 (171), SnTox5-Snn5 (172), SnTox6-Snn6 (173), and SnTox7-Snn7 (174). These are discussed in some detail in reference 249.

CWDEs

A diverse range of CWDEs, including xyloglucanases, polygalacturonases, glucanases, cellulases, and pectinases, are secreted by fungal pathogens. These enzymes are necessary for degradation of the main structural polysaccharide of plant cell walls, primarily cellulose, hemicellulose, and pectin (175–178). To study the direct contributions of CWDEs to the virulence of fungal pathogens during infection, genes encoding CWDEs have been disrupted and the effect on virulence has been evaluated in the mutants. Mutation in *B. cinerea* xylanase gene *Xyn11A* resulted in reduced ability of the mutants to infect grape berries and tomato leaves (179). In *C. gloeosporioides*, deletion of the pectate lyase gene *PelB* resulted in weak virulence on avocado fruit (180), while deletion of another pectate lyase gene, *CcpelA*, in *Colletotrichum coccodes* led to a substantial loss of virulence on green tomato fruit (181). Two cellulases from *M. oryzae*, belonging to glycosyl hydrolase families 6 and 7, were proved by RNA interference to contribute to penetration of the fungus into wheat leaf epidermis and for further invasion (182). Deletion of either endopolygalacturonase gene *Bcpg1* or *Bcpg2* in *B. cinerea* resulted in obvious reduction in virulence on bean and tomato leaves (183, 184).

Previously, it was generally accepted that the effect of CWDEs on pathogens' virulence was dependent on its enzymatic activity, and the lack of a clear pathogenicity phenotype in many CWDE mutants was attributed to redundancy. However, several recent studies have shown that at least some CWDEs may also act as virulence factors, either dependent or independent of their enzyme degradation activity. Several studies demonstrated that multiple CWDEs are detected as PAMPs by plant innate immune systems to induce responses (185, 186). For example, several endopolygalacturonases from *B. cinerea* can be recognized as PAMPs by the *A. thaliana* receptor-like protein RLP42 to promote immune responses (187), and one of these endopolygalacturonases, BcPG1, can induce defense responses independently of its enzymatic activity (188). The *B. cinerea* glucan 1,4-alpha-glucosidase BcGs1 can elicit systemic resistance against *B. cinerea*, *Pseudomonas syringae* pv. tomato DC3000, and tobacco mosaic virus (189). After Xyn11A was found to be necessary for virulence of *B. cinerea* (179), further studies showed that the enzymatic activity did not contribute to virulence, which was only affected by the necrosis-inducing activity of the peptide (190). The cutinase ScCut from *S. sclerotiorum* causes HR-like PCD in host plants and functions as an elicitor to trigger multiple defense responses in plants (191).

Effectors

Biotrophic effectors

Biotrophs depend on secreted effectors at several levels. Primarily, effectors are required during penetration to inhibit plant defense responses and/or to keep the host alive because of the lifestyle of biotrophic pathogens. Functional studies of several effectors have been conducted in the maize smut *U. maydis*. The effector Pep1 was first identified to be required for epidermal penetration (192). Later it was found that Pep1 can suppress the activity of the maize peroxidase POX12, which is involved in ROS generation (193, 194). The secreted effector Pit2 from *U. maydis* can inhibit the activity of maize cysteine proteases which can induce plant defenses of SA signal pathways (195), and mutation of *Pit2* in *U. maydis* causes severely attenuated tumors and virulence (196). The plant hormone SA is involved in a defense response against pathogens, especially biotrophic pathogens (197, 198). The chorismate mutase Cmu1, which is secreted into plant cells' cytoplasm by *U. maydis*, can reduce the levels of SA precursor chorismate, thus interfering with the synthesis of SA and promoting infection (199). The *U. maydis* effector See1 can interfere with the MAPK-triggered phosphorylation of maize SGT1, thus inhibiting its activity and resulting in modulation of reactivation of DNA synthesis in leaf cells, which is crucial for tumor progression (200). The *U. maydis* effector Tin2 interacts with and stabilizes maize kinase ZmTTK1, thus targeting the pathway of the maize secondary metabolite anthocyanin and promoting infection (201, 202).

In the *C. fulvum*-tomato pathosystem, for almost all identified effectors there is a corresponding tomato *Cf-R* trait (203). The presence of many cloned and functionally characterized avirulence genes and their corresponding *Cf* genes of tomato, and the emergence of many races overcoming newly introduced *Cf* genes, has made the *C. fulvum*-tomato interaction a good model system. Eleven effectors have been identified in *C. fulvum*, including Avr2, Avr4, Avr5, Avr9, Ecp1,

Ecp2, Ecp4, Ecp5, and Ecp6 (203, 204). However, only three of them have been functionally characterized. Avr4 is a chitin-binding protein that shields the fungal cell wall and protects the fungus against the deleterious effect of plant chitinases (14). The LysM domain containing effector Ecp6 is another chitin binding protein that sequesters chitin fragments released from fungal cell walls to prevent the elicitation of chitin-induced plant defenses (12). Avr2 is a cysteine protease inhibitor that binds to and inhibits the function of tomato Rcr3 cysteine protease activity (205). During coevolution, different pathogens that share the same host can independently develop different types of effectors that interact with the same host target important for plant immunity. For example, Avr2 of *C. fulvum* (fungi) (205, 206), EPIC1, and EPIC2B of *Phytophthora infestans* (an oomycete that causes late blight in potato and tomato) (207) and Gr-VAP1 of *Globodera rostochiensis* (yellow potato cyst nematode that can infect tomato) (208) can interact and inhibit tomato cysteine protease, $Rcr3^{pim}$.

The effector candidate CSEP0055 from the biotrophic pathogen *B. graminis* f. sp. *hordei* can interact with plant pathogenesis-related proteins PR1 and PR17, leading to inhibition of plant immunity (209). Silencing of *CSEP0055* by host-induced gene silence technology resulted in decreased frequency of fungal penetration sites (209). Silencing of RNase-like effectors BEC1011 and BEC1054 in *B. graminis* f. sp. *hordei* indicated their involvement in virulence, and complementation experiments showed that both of them may function inside the plant cell and that BEC1011 may be involved in interfering with pathogen-induced host cell death (210). The putative effector BEC4 from *B. graminis* f. sp. *hordei* can interact with ADP ribosylation factor-GTPase-activating protein and may interfere with defense-associated host vesicle trafficking (211). The yeast two-hybrid assay indicated that candidate secreted effector proteins CSEP0105 and CSEP0162 from *B. graminis* f. sp. *hordei* can interact with small heat shock proteins Hsp16.9 and Hsp17.5 of barley. Silencing of either of them resulted in decreased rates of haustorial formation. CSEP0105 contributes to fungal virulence by compromising the chaperone activity of barley Hsp16.9, which is involved in defense and stress responses against pathogen infection (212).

Hemibiotrophic effectors

Hemibiotrophic fungi require effectors to suppress the plant's immune system during their biotrophic stage, while a different set of effectors is needed to kill plant cells at the necrotrophic stage.

Prominent examples of effector interactions during the biotrophic phase in hemibiotrophs (but also in true biotrophs) include recognition of cell wall fragments. The LysM domain-containing pattern recognition receptor protein CEBiP in rice can recognize and bind the chitin oligosaccharides released from the cell walls of fungal pathogens directly, thus promoting chitin-triggered immune responses in rice cells (213, 214). The secretory effector protein Slp1 of *M. oryzae* also contains LysM domains, and it specifically accumulates at the interface of fungus and rice cells at the early stages of infection. Slp1 can compete with CEBiP for chitin oligosaccharides binding through the LysM domains, thus interfering with chitin-triggered immunity in the host. Consequently, virulence of *M. oryzae slp1* gene deletion strain Δ*slp1* was decreased compared to wild type (16). The cytoplasmic effector AvrPiz-t of *M. oryzae* contributes to virulence in rice plants that carry the R protein Piz-t. AvrPiz-t interacts with the rice RING E3 ubiquitin ligase APIP6 and inhibits its activity, thus suppressing oxidative burst and PTI in rice, which promotes virulence of *M. oryzae* (30).

The *M. oryzae* avirulence protein ACE1 contains a polyketide synthase and a nonribosomal peptide synthetase domain. ACE1 is exclusively upregulated in appressoria during the penetration stage (215–217). The unknown secondary metabolite synthesized by Ace1 is recognized by the R protein Pi33 in rice, which causes resistance. Mutations in the polyketide synthase domain of Ace1 led to the abolishment of Pi33-mediated avirulence activation (217). The predicted metalloprotease protein Avr-Pita from *M. oryzae* could elicit R protein Pi-ta-dependent resistance response in rice cells, but it did not contribute to virulence (218). The virulence-associated effector MC69 is involved in development of invasive hyphae after appressorium formation in rice leaf sheath, indicating its important roles in interaction with host plants. In addition, deletion of the MC69 orthologous gene in *C. orbiculare* also reduced the pathogenicity on both cucumber and tobacco leaves (219).The Pwl effectors Pwl1 and Pwl4 in *M. oryzae* can also act as Avr proteins that confer species-specific avirulence on weeping lovegrass (220, 221). An effector from *M. oryzae* that contributes to the necrotrophic phase is the snodprot1 homolog, MSP1, a member of the cerato-platanin family. MSP1 is secreted and contributes to the pathogenicity of *M. oryzae* on barley leaves. Application of recombinant MSP1 produced in *Escherichia coli* induces autophagy cell death, H_2O_2 production, and defense responses in rice leaves (31). Recently, a number of novel effectors were uncovered by genomic and transcriptomic analysis in the most

aggressive *M. oryzae* strain, 98-06. Five of the tested proteins can inhibit BAX-mediated apoptosis-like PCD in *Nicotiana benthamiana* leaves. The isolate-specific effector candidates Iug6 and Iug9 play significant roles in fungal propagation and pathogenicity, and overexpression of them suppresses the expression of defense-related genes in rice, suggesting their roles in the inhibition of host SA and ethylene signal pathways (28).

The tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*, which primarily grows in xylem vessels, secretes SIX (secreted-in-xylem proteins) effector proteins into the xylem to promote infection. To date, 14 SIX effector proteins (Six1-14) have been identified using mass spectrometry in *F. oxysporum* f. sp. *lycopersici*-infected tomato (222, 223). Several studies demonstrated that SIX1 (Avr3), SIX3 (Avr2), and SIX6 are required for pathogenicity on susceptible tomato lines (224–226). All three proteins also function as Avr proteins that trigger activation of resistance in the presence of the resistance genes; SIX4 (Avr1), Avr2, and Avr3 can trigger HR in plants harboring the R proteins I-1, I-2, and I-3, respectively (226). Additionally, Avr1 inhibits I-2- and I-3-mediated resistance (227), and recently, SIX6 was suggested to be involved in suppression of Avr2/I-2-mediated cell death when coexpressed in *N. benthamiana* (226, 228). Recently, a study showed that Avr2 interacts with SIX5, and the AVR2-SIX5 pair is required to activate I-2-mediated resistance in tomato (229).

Hemibiotrophic pathogens from *Colletotrichum* spp. can devastate many crop plants worldwide. To date, a number of genomic and transcriptomic studies in *Colletotrichum* spp. fungi have been conducted, and many candidate effectors were revealed during the interaction with host plants (48, 104, 127, 230, 231). The expression of the *CgDN3* gene encodes a secreted protein from *C. gloeosporioides* that was induced at the early infection stage, and the *CgDN3* deletion transformant failed to infect and reproduce on intact host leaves. Furthermore, CgDN3 was also involved in averting the hypersensitive-like response in host *Stylosanthes guianensis* (232). A necrosis-inducing secreted protein 1 (NIS1), which induces cell death in *N. benthamiana* leaves, was identified by functional screening of *C. orbiculare* cDNA, and the NIS1-induced cell death was suppressed by CgDN3 (233). The gene *CtNUDIX*, which encodes a nudix hydrolase domain containing effector from *C. truncatum*, is exclusively expressed during the late biotrophic stage, and CtNUDIX can elicit HR-like cell death in tobacco leaves. However, overexpression of *CtNUDIX* in *C. truncatum* and *M. oryzae* resulted in an HR-like response in infected plants and caused incompatibility with the host plants, indicating that *C. truncatum* utilizes CtNUDIX to elicit cell death to signal the transition from biotrophy to the necrotrophy phase (234). The host's nuclear targeted effector CgEP1 from the maize anthracnose pathogen *C. graminicola* is synthesized at the early stages of infection and contributes to disease development in maize. CgEP1 can bind to the promoter regions of several genes, thus transcriptionally regulating their expression to affect host immunity (235). Another effector of *C. graminicola* Cgfl (fungalysin metalloprotease), which specifically expressed at the biotrophic stage of infection, was identified with a role in virulence on maize leaves and roots. The maize leaves exhibited increased chitinase activity in the absence of Cgfl in the *in vitro* chitinase activity assays (236).

Necrotrophic effectors

Since necrotrophs extract nutrition from dead plant tissue, their virulence factors mainly aim to induce host necrosis to facilitate infection. The cerato-platanin BcSpl1 secreted by *B. cinerea* elicits the hypersensitive response in several host plants, and deletion of this gene results in reduced virulence in a variety of host plants, indicating that BcSpl1 is required for virulence (237, 238). Furthermore, BcSpl1 also induces systemic acquired resistance in tobacco to *P. syringae* and *B. cinerea* (239). *B. cinerea* could also manipulate the antagonistic effects between different immune pathways by utilizing exopolysaccharide β-(1,3)(1,6)-D-glucan to activate the SA signal pathway in tomato, thus inhibiting the jasmonic acid signaling pathway through NPR-1 and resulting in enhanced host susceptibility to *B. cinerea* (230). *B. cinerea* can also deliver virulent small RNA effectors into host plant cells to bind to *Arabidopsis* Argonaute 1 (AGO1), hijack the plant's RNA interference machinery, and selectively silence host immunity genes, thus suppressing host immunity and facilitating infection (241).

S. sclerotiorum secretes an integrin-like protein, SSITL, which functions as an effector to directly or indirectly suppress jasmonic/ethylene signal pathway-mediated immune responses in host plants at the early stages of infection. Silencing of *SSITL* caused a significant reduction in virulence and other phenotypic defects (242). Ss-Caf1 is another *S. sclerotiorum* small secretory protein with a putative Ca^{2+}-binding motif. Ss-Caf1 functions as a pathogenicity factor to trigger host cell death during the early stages of *S. sclerotiorum* infection and plays a significant role in the formation of infection cushion and sclerotial development (243). Another *S. scletoriorum* protein, SsCm1,

has high structural and functional similarity to the *U. maydis* effector Cmu1. One could speculate that this putative effector might serve a similar function as Cmu1 in *U. maydis* (199). A small secreted virulence-related protein, SsSSVP1 from *S. sclerotiorum*, induces plant cell death, and targeted silencing of SsSSVP1 causes decreased virulence. In addition, SsSSVP1 can interact with and hijack the activity of QCR8 (cytochrome b-c_1 complex subunit 8) by changing its subcellular localization from mitochondria to cytoplasm, hence disabling its biological functions. This causes abnormal plant phenotype and cell death (244). A new proteinaceous elicitor, sclerotinia culture filtrate elicitor 1 (SCFE1) from *S. sclerotiorum*, has been partially purified and induces typical PAMP-triggered immune responses through the RLP30/SOBIR1/BAK1-dependent signaling pathway in *A. thaliana* (245). A cysteine-rich secretory protein, SsCVNH from *S. sclerotiorum*, is essential for the virulence and sclerotial development, suggesting its diverse roles in host-nonspecific necrotrophic fungi (246). Two genes encoding necrosis and ethylene-inducing peptides, SsNep1 and SsNep2, have been cloned from *S. sclerotiorum*. Transient expression of them in tobacco leaves induces necrosis, and SsNep2 is involved in virulence (247).

CONCLUSIONS

As outlined in this article, fungi are a highly diverse group of plant pathogens with an enormous impact on agriculture. Fungi have a broad spectrum of lifestyles and high genetic flexibility, which allows pathogens to quickly invade new hosts, as well as to develop resistance to fungicide treatments or to break R-gene-mediated plant resistance. Therefore, fighting fungal diseases will remain a major challenge in plant pathology for the future. To develop new strategies to control plant pathogenic fungi, we need to increase our knowledge of fungal virulence mechanisms, of how pathogenic traits evolve, of the molecular basis of host adaptation, and in particular, of how crop resistance can be engineered in a more sustainable way.

Besides these applied aspects, plant pathogenic fungi are fascinating organisms that have developed a plethora of strategies to colonize a living plant. Studying the molecular mechanisms underlying these complex interactions will not only teach us about microbial virulence, but is also an excellent strategy to study cellular processes in plants. In particular, effector proteins are great molecular tools to study plant pathways, because they represent perfectly adapted probes to target specific cellular processes in the host. Beyond effector proteins, there is still little known about the functions of micro RNAs in fungal-plant interactions. Similarly, secondary metabolites are clearly an understudied field when it comes to biotrophic interactions. Finally, in past years, fungal-plant interactions were mostly studied in one-to-one situations, but this is very artificial because plants are always colonized by a complex microbiome in which multiple microbes influence each other and affect the plants in multiple ways. It is getting more and more evident that multitrophic interactions shape the outcome of pathogen infections. Therefore, future research on fungal plant pathogens will need to consider the effect of microbial communities on pathogen development and plant health.

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The Mutualistic Interaction between Plants and Arbuscular Mycorrhizal Fungi

35

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AN OVERVIEW OF MYCORRHIZAL INTERACTIONS

Mycorrhizal fungi are a heterogeneous group of diverse taxa associated with the roots of over 90% of all plant species, from liverworts to angiosperms. Although they can spend part of their life cycle in the rhizosphere, mycorrhizal fungi always associate with the roots of plants, including forest trees, wild grasses, and many crops, and colonize environments such as alpine and boreal zones, tropical forests, grasslands, and croplands. Both partners benefit from the relationship: mycorrhizal fungi improve the fitness of their host plants by influencing mineral nutrition and water absorption and by increasing tolerance to biotic and abiotic stresses. The host plant rewards the fungal symbiont with carbon compounds derived from the photosynthetic process (1).

Irrespective of their taxonomic position, mycorrhizal fungi develop an extensive hyphal network in the soil, the aptly named wood-wide web, which can connect whole plant communities and potentially grant the horizontal transfer of nutrients. Such an underground web has caused a paradigm shift in our knowledge of plant ecology by introducing the key role of below-ground microbes and opening the discussion on how they influence the composition and fitness of plant communities (2).

The term "mycorrhiza" is derived from the Greek words for "fungus" and "root": a beautiful linguistic rendering of such biological synergies. This does not in any way imply that mycorrhizal fungi only colonize roots; in fact, so-called basal plants lacking true roots also rely on mycorrhizal fungi for their nutrition and host the symbionts in other organs, such as the liverwort thallus. This feature is an evolutionary reminder of the first known mycorrhizal interactions: 450-million-year-old fossils of some of the first land plants display characteristic symbiotic fungal structures in their simple, prostrate shoots. In short, the evolution of mycorrhizal symbioses predates the appearance of roots, even if mycorrhizal fungi are currently restricted to roots in the vast majority of extant plants.

Mycorrhizal fungi can be divided into two major groups: aseptate endophytes such as Glomeromycota and septate Asco- and Basidiomycota. Usually, the classification of mycorrhizal interactions is based on plant anatomical traits and identifies two broad categories (Fig. 1): ectomycorrhizae, in which the fungus is restricted to the intercellular spaces of outer root tissues, and endomycorrhizae, in which fungi penetrate the living host cells (3). In ectomycorrhizae, which colonize trees and shrubs, hyphae remain extracellular, inducing important changes to root morphogenesis, while their presence leads to subtle modifications in epidermal or cortical cells. In endomycorrhizae, i.e., ericoid, orchid, and arbuscular mycorrhizae (AM), hyphae penetrate the root cells to establish an intracellular symbiosis. While AM are common to diverse plant taxa, ericoid and orchid mycorrhizae are restricted to the order *Ericales* and the family *Orchidaceae*, respectively. Such taxonomic and morphological diversity has stimulated the study of the colonization process in numerous host plants since the start of the 19th century. Indeed, new information generated by genomic sequencing, the use of mutants affected in their symbiotic capabilities, and live cell imaging has significantly changed our view of the colonization process (4).

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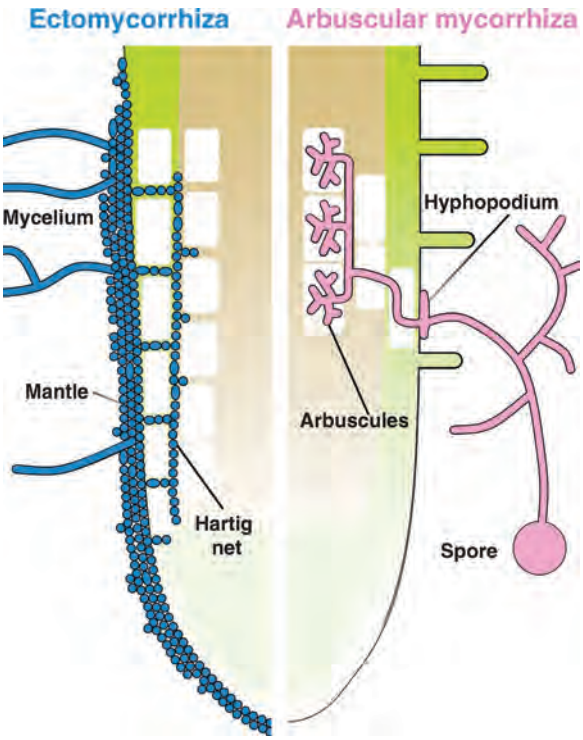


Figure 1 Root colonization in ectomycorrhizal (blue) and arbuscular mycorrhizal (pink) interactions. Ectomycorrhizal fungi envelop root tips with a thick mycelial mantle. From this mantle, intercellular hyphae generate the so-called Hartig net around epidermal cells. In the case of arbuscular mycorrhizae, the root tip is usually not colonized; hyphae developed from a germinated spore produce a hyphopodium on the root epidermis. Intraradical colonization proceeds both inter- and intracellularly, culminating with the development of highly branched arbuscules inside inner cortical cells. Reprinted from *Nature Communications* (3) with permission of the publisher.

The AM symbiosis is the most widespread plant-fungus mutualistic association. AM fungi are found in association with all plant phyla, including agriculturally relevant species, and play a crucial ecological role in the functioning of low-input environments. All AM fungi belong to the Glomeromycota phylum and are very specialized symbionts, as revealed by their obligate biotrophy. Currently there is broad interest in AM fungi because of their potential to support a reduction in the use of chemical fertilizers and pesticides and to positively impact sustainable crop production systems to feed a globally growing human population (5, 6). For these reasons this article will focus on the AM symbiosis, providing an overview of the current knowledge of the molecular mechanisms controlling this ancient and deeply intimate plant-fungus association.

ARBUSCULAR MYCORRHIZAE

AM fungi form a mutualistic symbiosis with the roots of ~80% of plant species in natural and agricultural systems and are considered a central component of the plant microbiota.

AM fungi have the unusual ability to grow in two extremely different niches: the soil and the lumen of plant cells. In soil, the extensive hyphal network has been estimated to reach a density of 100 m/cm^3 (7). This hyphal network can acquire water and nutrients with great efficiency and reach a volume of soil that is inaccessible to roots alone (8). The presence and activity of AM fungal mycelia also directly influence the physico-chemical properties of soil (9). Inside their host plant, AM fungi penetrate single cells of the root cortex, where they develop a structure that is the distinctive feature of this association: the arbuscule (Fig. 2). Named from the Latin word for bush or small tree, each arbuscule results from repeated branching of a single hypha in the lumen of a parenchymatic cell from the inner root cortex (10). With their impressive surface-volume ratio, arbuscules are considered the major site of nutrient exchange (11).

As obligate biotrophs, AM fungi strictly depend on their plant hosts for both growth and reproduction (3, 12). Up to 20% of the photosynthesis products of terrestrial plants is consumed by AM fungi (13). Interestingly, the carbon flow from the plant to the fungus seems to be proportional to the amount of phosphate that the fungus returns to its host (14). Consequently, while the beneficial effects of AM fungi become evident when the nutrient and water supplies are limited, root colonization decreases in soils with abundant nutrients.

The AM interaction also supports important ecological services such as an increase of soil quality (15) and of biodiversity of the associated plant communities (16). Additionally, many studies have highlighted the positive influence of the AM symbiosis on plant responses to biotic (17, 18) and abiotic stresses such as drought, salinity, and heavy metal contaminants (6, 19, 20).

Classification and Phylogeny of AM Fungi

AM fungi have existed and coevolved with plants for at least 450 million years (21) as shown by fossils (22). Due to their importance in nutrient transfer, they are supposed to have played a crucial role during land colonization by plants. The widespread occurrence of AM fungi in plants from most parts of the world, particularly in the tropics, was acknowledged in the 19th century (23, 24). In 1844 the Tulasne brothers described the first AM fungal species, *Glomus microcarpum* and *Glomus macrocarpum* (25). In 2001 Schüßler and col-

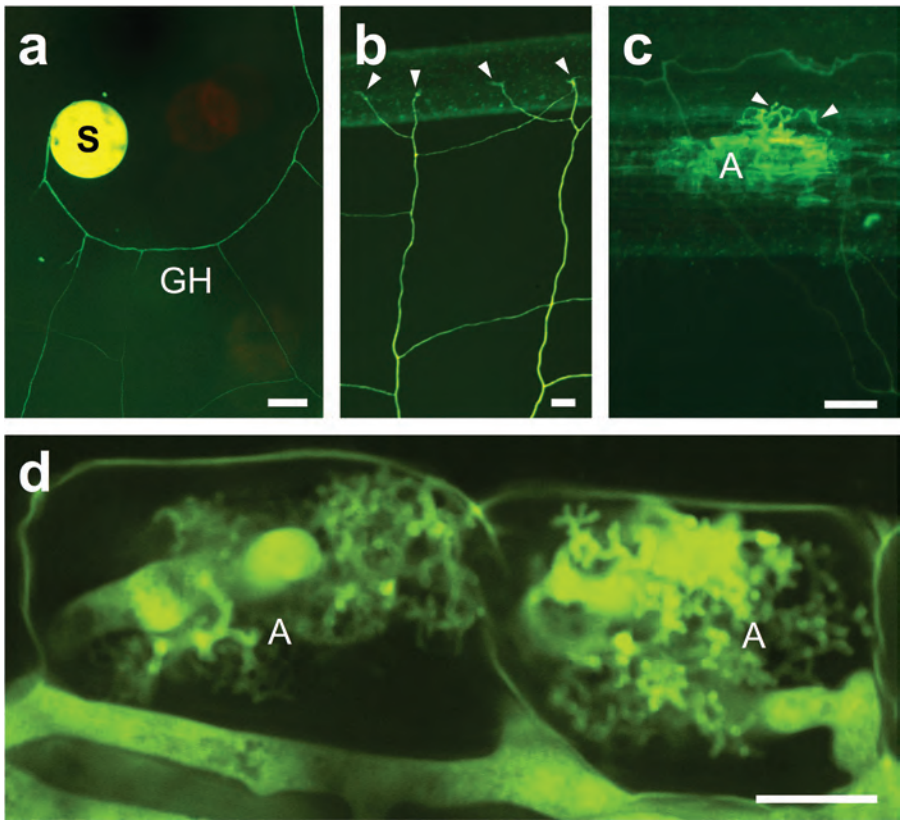


Figure 2 Fluorescence micrographs of different stages in the life cycle of the AM fungus *Gigaspora gigantea*. (**a**) A spore (S) and the germination hyphae (GH) show strong cytoplasmic autofluorescence. (**b**) Hyphopodia (arrows) on the surface of a host root give rise to single infection units (**c**) with several arbuscules (A) in the inner root cortex. (**d**) A high magnification from a root longitudinal section showing two arbuscules in adjacent cortical cells. Bars = 100 μm (a–c), 25 μm (d); fungal fluorescence was excited with 380–405 nm UV light.

leagues grouped AM fungi within a new monophyletic phylum, the Glomeromycota, distinct from the Zygomycota, where they had previously been placed (26).

The phylum Glomeromycota is currently represented by about 250 described species (4), although molecular analyses suggest a broader diversity (27). A recent survey of the global distribution of these plant symbionts showed that AM fungal communities reflected local environmental conditions and the spatial distance between sites. However, despite AM fungi apparently possessing limited dispersal ability, 93% of taxa were found on multiple continents and 34% on all of the six surveyed continents (28).

Before the advent of molecular techniques, the identification of AM fungi was based on the microscopic examination of spores. These are roundish, from about 30 to 400 μm in diameter and with features with taxonomic value such as color, dimension, cell wall structure, presence of septa, and connection to sustaining hypha. However, morphological similarities do not always reflect phylogenetic relationships. The development of PCR-based approaches and advances in molecular analyses led to novel identification rules (26, 29). Since then, the taxonomy of Glomeromycota has been subjected to several changes, and it is still largely open to discussion. On the basis of rRNA gene phylogeny Glomeromycota were described as a sister group of Ascomycota and Basidiomycota (26, 29). However, phylogenetic reconstructions based on mitochondrial (30–32) or protein-coding nuclear (33) sequences suggest a closer relationship with Mortierellales or Mucorales (Mucoromycotina). Recently, on the basis of the complete genome sequence of *Rhizophagus irregularis* (34, 35), Glomeromycota were again phylogenetically placed closer to Mucoromycotina (see later in this article). Indeed, Mucoromycotina, a basal group of fungi characterized by a coenocytic mycelium, is now considered a sister group of Glomeromycota (36). Interestingly,

Mucoromycotina form functional mycorrhiza-like associations with basal plant lineages (37), suggesting that the symbiotic options available to the first plants emerging onto dry land were more varied than previously thought (38). It now appears likely that the last common ancestor of both fungal groups thrived in primeval soils long before plants colonized the land (36).

Biological Features, Genome Organization, and Genetics of AM Fungi

AM fungi are also intriguing from a cellular and genetic point of view. They display many unusual biological features in addition to their obligate biotrophism: spores and coenocytic hyphae contain thousands of nuclei in a common cytoplasm, and no uninucleate life stage is known to occur. This makes classical genetic approaches challenging (12, 36). Depending on the species, a single spore contains from 800 to about 35,000 nuclei (39). Single spores are populated by flows of unrelated nuclei streaming from the mycelium, rather than by the replication of one or a few nuclei within the developing spore (40).

The concept of species is poorly defined in this group of organisms, since they show a high degree of genetic variability. For example, the intrasporal variability of the internal transcribed spacer ribosomal region can range between 6% for *Gigaspora margarita* (41), 18% for *Glomus intraradices* (42), and over 20% in other *Glomus* "*intraradices*-like" strains (43).

In spite of being among the oldest living terrestrial organisms, AM fungi surprisingly appear to have lost sexual reproduction, because no sexual cycle has ever been described.

The genetic organization of coexisting nuclei in the Glomeromycota (i.e., coenocytic organisms) has been a major source of debate for the past 15 years, with two opposite hypotheses being supported within the research community: heterokaryosis (44) and homokaryosis (45), which support, respectively, the presence or the absence of internuclear genetic divergence (46). Furthermore, the possibility that AM fungi contain a uniform population of nuclei characterized by intranuclear polymorphism has also been proposed (47). Heterokaryosis may originate by hyphal anastomosis between genetically different mycelia or by the accumulation of mutations. Hyphal anastomosis and the exchange of nuclei have indeed been observed in a few fungal taxa (48, 49); nevertheless, such events have only been observed between distinct isolates of the same species from one sampling area (50), but not between isolates from different geographical areas (51). These opposing hypotheses have been reconciled by recent analyses of complete genome sequences of AM fungi (see next paragraph).

A further increase in the genetic complexity of AM fungi is evidenced by the presence of endobacteria and viruses. Many AM fungi harbor endobacteria in their hyphae and spores; two types of endobacteria are known in Glomeromycota: rod-shaped Gram-negative endobacteria, limited in distribution to members of the *Gigasporaceae* family, and coccoid Mollicutes-related endobacteria, distributed across different lineages of AM fungi (52). The genomes of a few endobacteria have been sequenced: all are characterized by a limited size (0.5 to 1.8 Mb), which is consistent with complete dependence on their fungal host and their inability to grow in pure culture (53–55). So far, the biological role has only been investigated for "*Candidatus* Glomeribacter gigasporarum," the rod-shaped bacterium hosted by *G. margarita*. The endobacterium enhances fungal sporulation, bioenergetic capacity, ATP production, and ability to detoxify reactive oxygen species. Endobacteria also appear to enhance fungal responsiveness to strigolactones (SLs), root-exudated signals that act as hyphal branching factors for AM fungi. Overall, this endosymbiosis improves the fungal ecological fitness by priming mitochondrial metabolic pathways and giving the AM fungi more tools to face environmental stresses (56, 57).

Our knowledge of mycoviruses of AM fungi is still very limited (58, 59). In one case a biological function has been reported: the presence of the virus led to the production of more spores and increased stimulation of plant growth (58). Altogether, these studies underline the importance of these additional genetic components of AM fungi, because such components can contribute to the symbiosis.

The First Genome Project Dedicated to an AM Fungus

Extensive efforts were made to sequence the first genome of an AM fungus. Two independent research groups (34, 35) published the genome sequences of the same *R. irregularis* isolate DAOM197198 (60). By sampling the extraradical hyphae of mycorrhizal hairy root cultures, Tisserant and colleagues obtained an assembly of 101 Mb out of an estimated genome size of 153 Mb (34). This assembly, although highly fragmented, is believed to include almost all the protein-coding genes (23,561 high-confidence gene models) and places the genome of *R. irregularis* among the largest fungal genomes sequenced to date, along with those of obligate biotrophic powdery mildews (61) and the ectomycorrhizal symbiont *Tuber melanosporum* (62).

To specifically address the issue of the heterokaryotic nature and to determine the extent to which nuclei differ from each other, Lin and colleagues performed a *de novo* genome sequencing of individual nuclei collected from crushed spores (35). Their comparative analysis revealed a surprisingly low level of polymorphism: >99.97% of the aligned genome sequences were identical in different nuclei. The genome organization of this strain is therefore considered basically homokaryotic. By contrast, within a single nucleus, the 45S rDNA repeat unit—in particular, the internal transcribed spacer region—turned out to be highly diverged, and the relative abundance of alleles appeared to differ between nuclei. Genome annotation resulted in 27,392 protein-coding gene models representing 30,003 putative transcripts with a high level of putative/predicted (retro-)transposable elements (35), findings consistent with those of Tisserant et al. (34).

The gene repertoire of *R. irregularis* mostly overlaps with the repertoire of sequenced Mucoromycotina species (34, 35), and phylogenomic analyses demonstrated that Glomeromycota are more closely related to Mucoromycotina than to their postulated sister Dikarya (35).

The obligate biotrophy of AM fungi is not mirrored by any drastic loss of metabolic complexity in central metabolism. One striking genomic feature is the lack of genes encoding plant cell wall-degrading enzymes; the same situation has been observed in obligate biotrophic pathogens (61) and ectomycorrhizal symbionts (62).

More recently, the *in silico* genome analysis of five *R. irregularis* isolates (63) has highlighted substantial genetic diversity among isolates. Two isolates contained a stable population of two dominant divergent haploid nuclei (a unique dikaryon-like condition), while the other three isolates only displayed one dominant genotype (63). *R. irregularis* therefore appears to develop both homokaryotic and heretokaryotic mycelia, even if the modes by which this differential genomic pattern arises remain unclear. In addition, the identification of a putative mating type locus suggests the existence of cryptic sex-related processes (34, 63). Besides reconciling the previous contrasting results, such novel data indicate that conventional modes of reproduction—including mating—may exist in this lineage, which can suggest approaches to deliver genetic improvement of AM strains.

The Colonization Process

The establishment of the AM symbiosis requires a succession of well-characterized developmental steps (Fig. 3) (3). In advance of direct plant-fungus contact—the so-called presymbiotic stage—diffusible molecules mediate reciprocal recognition between the two symbionts. Then the fungal hyphae contact the root epidermis, where they form adhesion structures called hyphopodia; these precede root entry and mark the initiation of the symbiotic phase. In response to these chemical and physical stimuli, the contacted epidermal cell develops an intracellular accommodation structure, called the prepenetration apparatus (64), which drives fungal penetration and guides hyphal passage across the epidermal cell and toward the inner root tissues. Within the root, hyphae grow inter- and intracellularly to reach the deepest cortical cells, where arbuscules develop (65). As root colonization proceeds, the AM fungus explores the soil, developing an extensive hyphal network, the extraradical mycelium, which also can produce a new generation of asexual spores.

Host plants control each step of symbiosis development, leading to a precise synchronization of fungal and plant developmental processes (65, 66). Over the past decade, the molecular components controlling AM colonization have been intensively studied in angiosperms (11, 65) and more recently in basal land plants (67, 68).

Presymbiotic signaling

AM fungi perceive the vicinity of a host via root-exuded molecules that induce spore germination and hyphal branching (69, 70). The most-studied plant symbiotic signals are carotenoid-derived phytohormones called strigolactones (SLs) (71), which have a primary role in plant development (72). AM fungi sense SLs in root exudates at concentrations as low as 10 nM. Fungal responses to GR24, a synthetic molecule commonly used to study SL actions, include the enlargement of mitochondria, a rapid increase in ATP, and NADH and nuclear proliferation (73–76). GR24 exposure also causes a sharp increase in Ca^{2+} concentration in the fungal cytoplasm (77). Although fungal SL receptors remain unknown, such observations suggest that SLs are perceived via a Ca^{2+}-mediated signaling pathway and trigger a cellular and molecular prelude to root colonization (74, 75, 78).

AM fungi also release signal molecules that trigger plant symbiotic responses (79), including transcriptional regulation, nucleus-associated Ca^{2+} signals, starch accumulation in roots, and lateral root formation (80–86). Repeated oscillations in nuclear Ca^{2+} concentration (spiking) have been observed in the root epidermal cells contacted by AM fungal hyphopodia, but also when the same cells were treated with exudates from germinated AM fungal spores (83). Similarly, the expression of the early symbiotic gene *ENOD11* in *Medicago truncatula*

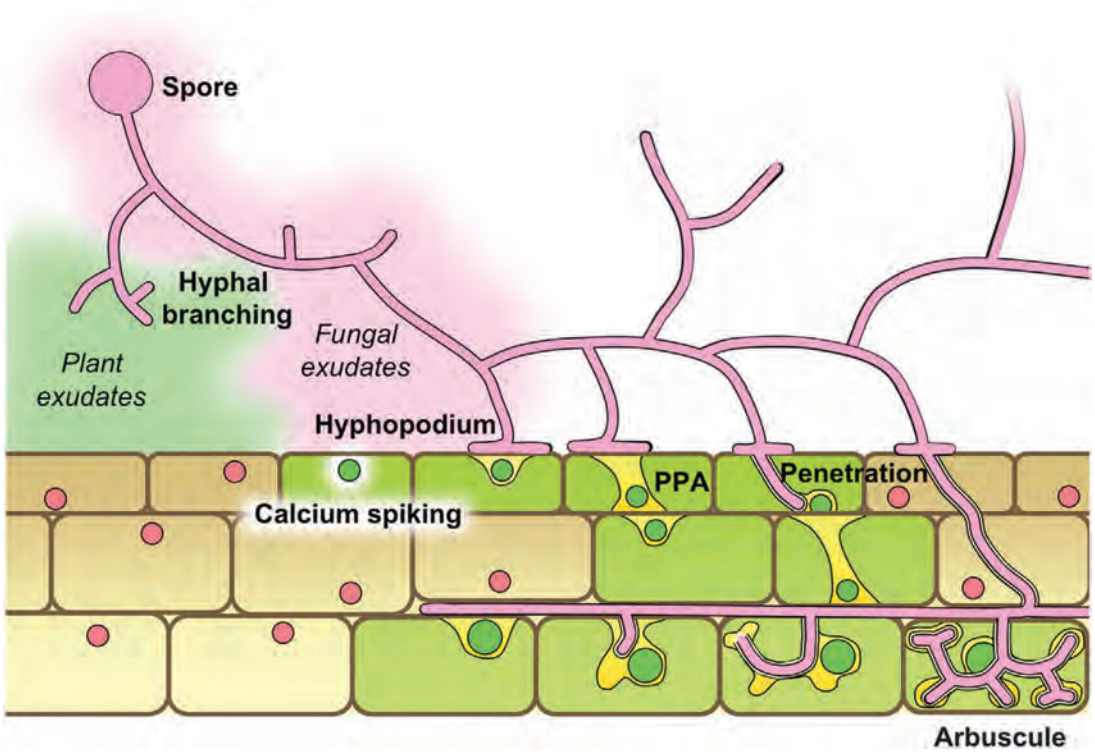


Figure 3 Root colonization by AM fungi. Spore germination generates a short explorative mycelium. The perception of root exudates induces repeated hyphal branching, increasing the probability of direct contact between the symbionts. Concurrently, fungal exudates are also released and activate the common symbiotic signaling pathway in root cells. Signal transduction includes nuclear-associated calcium signals (spiking) and leads to the activation of cellular and transcriptional responses (green cells and nuclei). Plant-fungus contact is followed by the formation of an adhering hyphopodium on the root surface. The contacted epidermal cell then assembles a prepenetration apparatus (PPA), a broad cytoplasmic aggregation (yellow) responsible for the exocytotic biogenesis of the symbiotic interface compartment, where the intracellular hypha is hosted. Root colonization proceeds through the epidermis into the inner cortical cells with a PPA-like process. Intercellular hyphae can also develop along the root axis. Eventually, highly branched arbuscules develop in the lumen of inner cortical cells, deploying an extensive surface for nutrient exchange. Reprinted from *Nature Communications* (3) with permission of the publisher.

is upregulated upon both fungal contact (87) and the perception of fungal exudates (80).

Different *N*-acetylglucosamine oligosaccharides have been characterized in AM fungal exudates as bioactive molecules responsible for such plant responses. They include tetra- and penta-chito-oligosaccharides (CO4 and CO5) (86) as well as lipo-chito-oligosaccharides, which are very similar to nodulation (Nod) factors released by nitrogen-fixing rhizobia (85). When applied as purified molecules, such chitin derivatives mimic the perception of fungal exudates in the host roots, including nuclear Ca^{2+} spiking (85, 86) and the regulation of symbiosis-related genes (80, 85, 88). Interestingly, the release of CO4 and CO5 in *R. irregularis* exudate is boosted upon GR24 treatment (86), suggesting the existence of a positive loop between plant and fungal signal perception and production of these oligosaccharides.

The study of plant signaling mechanisms involved in the perception of AM fungal signals has been developed in legumes such as *M. truncatula*, largely following the research on rhizobial Nod factor signaling. Such comparative investigations have revealed the existence of a so-called common symbiotic signaling pathway (CSSP), which includes several genes that are essential for both symbioses (89, 90). Evidence that the same genes are also involved in diverse symbiotic, pathogenic, and parasitic plant interactions is accumulating (91).

The CSSP starts on the plant cell membrane, with a malectin-like domain leucine-rich repeat receptor-like

kinase (known as SYMRK, in *Lotus japonicus*). SYMRK forms a complex with Nod factor receptors NFR1 and NFR5 and is believed to interact also with the so far unidentified receptor(s) for AM fungal signals (90). In its cytoplasmic domain, SYMRK also interacts with a MAP kinase kinase (92) and HMGR1, a 3-hydroxy-3-methylglutaryl-CoA reductase involved in mevalonate synthesis. Indeed, mevalonate has recently been demonstrated to trigger downstream symbiotic responses such as nuclear Ca^{2+} spiking and ENOD11 expression (93).

All the remaining CSSP proteins that have currently been identified are localized to the nucleus. They include three nucleoporins (NUP85, NUP133, and NENA [94–96]) possibly involved in nuclear targeting of CSSP actors, as well as the ion channel CASTOR/POLLUX and the SERCA-type Ca^{2+}-ATPase MCA8. Both these latter proteins localize to the nuclear envelope (97, 98) and are essential for nuclear Ca^{2+} spiking (98–101). The nuclear envelope lumen is considered to be the site where Ca^{2+} is stored and released during symbiotic Ca^{2+} signaling. The Ca^{2+}- and calmodulin-dependent protein kinase CCaMK localizes to the nucleoplasm and is composed of a serine/threonine kinase domain, a calmodulin-binding domain, and three Ca^{2+}-binding EF-hand domains (102, 103). Upon an increase in Ca^{2+} concentration, CCaMK is subject to a complex conformational change (102, 104, 105). When active, CCaMK regulates gene expression through its interacting partner, CYCLOPS (106–108), and the action of transcription factors such as NSP1 and NSP2 (109–112), NIN (113, 114), and reduced arbuscular mycorrhiza 1 (RAM1) (89, 115).

The exchange of chemical signals that mediate reciprocal recognition probably becomes more intense as soon as a hyphopodium adheres to the surface of the root epidermis. Hyphopodium differentiation depends on plant cell wall-bound signals as shown by the seminal studies of Giovannetti et al. (116) and Nagahashi and Douds (117). Only recently, though, monomeric cutin has been proposed to be responsible for hyphopodium differentiation. This deduction comes from the observation that RAM1 activation increases the expression of RAM2, a glycerol-3-phosphate acyl transferase involved in the biosynthesis of cutin precursors (115, 118).

Interestingly, in a recent paper Gutjahr et al. (119) identified loss of responsiveness to AM fungi in a rice mutant, which was also mirrored by the absence of physical contact and of characteristic transcriptional responses to AM fungal diffusible signals. The gene responsible for the loss of symbiosis, *DWARF 14 LIKE* (*D14L*), encodes an alpha/beta-fold hydrolase that is a component of an intracellular receptor complex involved in the detection of the smoke compound, karrikin. Thus, D14L seems to be required to support initial colonization events by AM fungi. Overall, these results reveal a novel plant recognition strategy for AM fungi and envisage the existence of an additional signaling molecule, the D14L ligand.

Host cell colonization

As soon as a hyphopodium develops on the root surface, the nucleus of the underlying epidermal cell moves toward the fungal contact site, then migrates to the opposite side of the cell traversing the lumen and partially displacing the vacuole. Concurrently, a broad, columnar cytoplasmic aggregation assembles between the nucleus and the fungal contact site. This aggregate is rich in endoplasmic reticulum, cytoskeleton, Golgi stacks, and secretory membranes and constitutes the so-called prepenetration apparatus, or PPA (Fig. 3) (64, 66, 120). Such features characterize the PPA as a broad exocytotic process finalized at the construction of the novel membrane domain—an extension of the host plasmalemma—where the fungus will be hosted: the perifungal membrane which envelops all intracellular fungal structures (120). Only after the PPA is fully deployed, a penetrating hypha develops from the hyphopodium, crosses the epidermal cell wall, and enters the cell lumen, strictly following the PPA route (64). PPAs are not observed in plants that lack CSSP genes such as *dmi2* or *dmi3* (64). Furthermore, constitutive expression of an active CCaMK variant induces cytoplasmic aggregates that resemble a PPA (121). Consequently, one key function of the CSSP is the activation of the cellular program responsible for fungal hosting (11).

PPA formation is not limited to epidermal cells, where the fungus starts its intracellular development, but is also observed in outer and inner cortical cells in preparation for arbuscule formation (66). Cortical cells that are preparing to harbor an arbuscule display the most extensive PPAs: here, the cell membrane invagination does not envelop a single hypha but progressively expands to line each of the fine branches that can fill up most of the cell lumen (10). The perifungal membrane around intracellular hyphae or the periarbuscular membrane (PAM) around arbuscules (65) outlines the so-called symbiotic interface, the novel cell compartment where the fungus is hosted and where most of the signal and nutrient exchanges are believed to occur (122, 123). In line with this, the PAM comprises a specific subset of membrane-associated proteins (124). Though the signal that induces branching and differentiation of arbuscules is currently unknown, several

plant genes required for arbuscule development and/or function have been identified, including Vapyrin (125), two vesicle-associated membrane proteins (126), EXO70I (127), proteases (128, 129), a proton ATPase (130, 131), ATP-binding cassette transporters, stunted arbuscule and stunted arbuscule 2 (132), and phosphate transporters (133, 134).

Interestingly, trafficking of the symbiotic phosphate transporters and ATP-binding cassette transporters to the PAM requires gene expression coincident with arbuscule branching (135), leading to the hypothesis that PAM construction is achieved by synchronizing two cellular and molecular processes: the massive reorientation of exocytosis toward the developing PAM and the transcription of specific genes encoding for PAM-resident proteins (11, 135). Because transcriptional control seems crucial to ensure the correct protein composition of the PAM, plant transcription factors active in the AM symbiosis have also been characterized. So far, transcription factors required for AM symbiosis include CYCLOPS (108, 136), the gibberellin repressor protein DELLAs (137, 138), RAM1 (115), required for arbuscule development 1 (RAD1) (139), MtERF1 (140), and DELLA-interacting protein 1 (141). Recent results suggest a model where DELLA proteins regulate arbuscule development through modulation of RAM1 and RAD1 that in turn regulate genes required to support arbuscule branching (142).

Arbuscules are ephemeral structures that collapse and degenerate approximately 2 to 3 days after maturity (11, 143), while the host cell regains its previous organization and can undergo a new round of colonization.

The correct formation and functioning of an arbuscule are also expected to be under fungal control; however, the functional studies of the fungal partner are very few. Kloppholz et al. (144) discovered the first AM fungal effector, named secreted protein 7. Effector proteins are secreted by plant-colonizing microbes and are generally thought to promote compatibility or to suppress plant defense responses by interfering with metabolism or signaling pathways (145, 197). In particular, secreted protein 7, which is secreted into the host cell and localizes to the plant nucleus, counteracts the plant immune response by interacting with the pathogenesis-related transcription factor ethylene response factor 19 (144). Although their mechanisms of action have not been elucidated yet, two additional fungal genes have been recently identified with a putative role in the accommodation of fungal structures in the root (146, 147).

While such targeted investigations have started shedding light on the cellular and molecular mechanisms that act inside each host cell, transcriptomic and genomic studies suggest that these are just the first steps of the characterization of fungal effectors and their function in AM (148, 149).

The Transfer of Nutrients

Nutrient uptake and transfer to the host plant are the most documented roles of AM fungi. The extraradical mycelium acts as an extension of the root system, taking up phosphate (P), nitrogen (N), sulfur (S), and trace elements from the soil and delivering them to the host plant via the intraradical mycelium (8). The PAM is considered to be the site where this symbiotic transfer occurs: mineral nutrients released in the interface compartment are captured by PAM-bound plant transporters that translocate them to the host cell cytoplasm (150).

Phosphorus

Two pathways contribute to inorganic phosphate (Pi) uptake in mycorrhizal plants: a direct pathway by the root epidermal cells and a mycorrhizal pathway via AM fungi (151, 152). AM fungi are capable of significantly improving the uptake of Pi ions, which are characterized by low mobility and availability in soil. By using radioactive P, Smith and Smith (8) found that in mycorrhizal plants most of the P delivered to the plant came from the fungus and that the direct pathway was almost inactive. Indeed, depending on the plant and fungal species, AM fungi can contribute from 20 to 100% of the plant P uptake (153).

The mycorrhizal pathway involves initially the fungal uptake of soluble Pi from the soil. This is mediated by $Pi:H^+$ transporters, which have been described in *Diversispora epigaea* (154), *R. irregularis* (155), and *Funneliformis mosseae* (156). However, a role remains to be clarified for the putative $Pi:Na^+$ transporters RiPT1 and RiPT2, recently identified in the *R. irregularis* genome (152).

Within the extraradical mycelium, Pi can supply the metabolically active Pi pool (used for the biosynthesis of phospholipids, DNA, RNA, and proteins) or rapidly accumulate in vacuoles in the form of long-chain or short-chain polyphosphates (polyP) (157, 158), presumably through the action of the polyP polymerase/vacuolar transporter chaperone complex (148). PolyP is considered the major Pi store in hyphae as well as the main form of Pi translocation over long distances within hyphae (159). Indeed, polyP accumulation in the extraradical mycelium mirrors equivalent Pi uptake from the soil (160). Interestingly, polyP translocation toward the host is mediated by the activity of a fungal

aquaglyceroporin, which is highly expressed in the intraradical mycelium and is responsible for water transport across the plasma membrane (161). These findings provide novel insights on the mechanisms involved in the directional P transport toward the roots, and they highlight a key role of host transpiration and fungal aquaporins. PolyP degradation in the intraradical mycelium, possibly by vacuolar endopolyphosphatase and exopolyphosphatase activities, sustains Pi flux from the fungus to the apoplastic interface compartment (148, 162).

On the plant side, the activation of the mycorrhizal pathway is mirrored by the downregulation of plant Pht1 (H^+-dependent) transporters located in root epidermal cells, such as *M. truncatula* phosphate transporters MtPT1 and MtPT2 (163), and the upregulation of mycorrhiza-inducible Pth1 transporters (152 and references therein). Some of the latter are mostly or exclusively expressed in arbusculated cells. Among them, MtPT4 localizes to the PAM surrounding the arbuscule branches (124, 164). Interestingly, the two mycorrhiza-inducible Pi transporters, *MtPT4* and *LjPT4*, are expressed in the root tips of noncolonized plants, suggesting that they play a role in the Pi-sensing machinery of root tips (165).

Nitrogen

Although the impact of AM symbiosis on plant N uptake is not as clearly defined as that of Pi, there is increasing evidence for the existence of an N pathway through the fungal hyphae to the host plant, in spite of the contribution of AM fungi to the plant total N nutrition varying considerably depending on the context (8, 158, 166). Nitrogen is found in soil in both organic and inorganic compounds, and plants use all of them. The former include simple molecules such as urea, amino acids, amines, and peptides and complex molecules such as proteins, while inorganic N compounds are mainly represented by nitrate (NO_3^-) and ammonium (NH_4^+). In soils where N is limited or poorly mobile, due to drought or acidity, the contribution of the AM fungus to plant N nutrition can be considerable (152, 167), ranging between 24 and 42% of a plant total N content (168).

A few mechanisms of N uptake and transfer in the AM symbiosis have been recently described (166). Extraradical hyphae preferentially take up NH_4^+, which is energetically less costly than alternative N sources such as NO_3^- and amino acids. López-Pedrosa et al. (169) demonstrated that *GintAMT1*, a gene encoding for a high-affinity NH_4^+ transporter (AMT) in the AM fungus *R. irregularis*, is expressed in the extraradical mycelium. A second *R. irregularis* AMT has been characterized (170). *GintAMT1* and *GintAMT2* are differentially expressed during the fungal life cycle and in response to N. In contrast to *GintAMT1*, *GintAMT2* transcript levels are higher in the intraradical than extraradical hyphae.

Inside the extraradical mycelium, N compounds are converted into amino acids, mainly arginine (171, 172). Arginine is then translocated to the intraradical hyphae within tubular vacuoles and then reconverted into inorganic N compounds by the sequential enzymatic activity of arginase and urease: NH_4^+ is the most likely form of N transferred from fungus to plant (173, 174). It has been proposed that arginine binds to the negatively charged polyP and that both could move together within the hyphae (171, 175). The eventual transfer of NH_4^+ from the apoplast to the plant cells probably relies on NH_4^+ transporters sitting on the PAM (176–178). Remarkably, mutations in AM-specific Pi and NH_4^+ transporters have an impact on intraradical fungal development and arbuscule lifespan (179, 180). It has been speculated that these transporters not only deliver nutrients to the plant cells, but also trigger signaling processes that control arbuscule maintenance (180).

Even though the flux of mineral nutrients within the periarbuscular space is assumed to be directed toward the plant cell, fungal Pi (181, 182) and NH_4^+ transporters (170) are expressed in arbuscules. This finding suggests that the fungus may recover nutrients from the periarbuscular interface as a mechanism to control the amount of nutrients delivered to the host.

As well as taking up inorganic N, AM fungi also appear to obtain N from complex organic material (183–186). Such a process probably involves, among other transporters, an amino acid permease (AAP). A fungal AAP (GmosAAP1) has been characterized in *F. mosseae*. Since *GmosAAP1* is expressed in the extraradical mycelium and upregulated upon exposure to organic nitrogen (187), this gene may play a role in the first steps of amino acid acquisition from the soil. Since short peptides can represent a greater proportion of N in soils than free amino acids, it is notable that AM fungi also possess functional dipeptide transporters such as RiPTR2 (188). In yeast complementation assays, RiPTR2 allowed the uptake of several dipeptides such as Ala-Leu, Ala-Tyr, and Tyr-Ala. *RiPTR2* is expressed in the extraradical hyphae, suggesting a role in the uptake of organic N from soil; however, a stronger expression is consistently observed in the intraradical phase (188). This finding points to a function for this transporter in the mobilization of organic N in mycorrhizal roots.

Despite all the interesting findings, many critical questions about N transport through the fungal hyphae and across the mycorrhizal interface are still unanswered and should be addressed in future studies (166).

Other mineral nutrients

In addition to the improvement of plant N and P nutrition, physiological studies also have highlighted a role for AM fungi in enhancing the absorption of other ions such as sulfur, potassium, or different secondary macro- and microelements (1, 152).

Sulfur (S) is a key macronutrient for plant growth, development, and response to several stresses. Casieri et al. (189) observed an increased S content in mycorrhizal compared to nonmycorrhizal plants and the upregulation of two S transporters (*MtSULTR1.1* and *MtSULTR1.2*) in *M. truncatula*. More recently, an *L. japonicus* transporter (LjSultr1;2), specifically involved in sulfate uptake from arbuscules, has been identified (190).

Despite the importance of potassium (K^+) for plant growth, the contribution of AM symbiosis to plant K^+ nutrition has only occasionally been studied. It appears that plant K^+ nutrition is improved by mycorrhization, especially under K^+ limiting conditions. Moreover, this improvement could affect abiotic stress tolerance, P homeostasis maintenance, or exclusion of soil contaminants such as radiocaesium (191 and references therein). The characterization of genes involved in the transport and metabolism of K^+ and other mineral nutrients is required before a comprehensive map of the transportome of arbuscular mycorrhizae can be developed.

Carbon

In exchange for plants' improved access to nutrients, AM fungi take advantage of carbon compounds of plant origin, consuming between 10 and 30% of the plant photosynthates (192). The transcriptional regulation of genes involved in sucrose transport has been reported in several plant-fungus combinations (152 and references therein), although more efforts are required to clarify which plant sucrose transporters and regulatory mechanisms are active in sucrose partitioning during mycorrhization. On the fungal side, only one high-affinity monosaccharide transporter (MST), probably responsible for C uptake from the interface compartment, has been described (193). The gene *MST2* is highly expressed in arbuscules and intercellular hyphae. The high affinity and transport capability for xylose residues suggest that the use of derivatives from plant cell wall polymers could be an additional source of C. Interestingly, *MST2* is also expressed in extraradical hyphae, which can take up glucose and xylose, suggesting a partial metabolic independence of AM fungi from host plants.

For full mutualism to occur, a functional linkage between C and P exchange, under fine control by both partners, is likely to be in place. Recent studies have demonstrated that a strategy of reciprocal rewards rules AM interactions: in the presence of multiple partners, the most beneficial partner is rewarded with the majority of resources (14, 194). A similar mechanism also appears to regulate C and N exchange (175). These striking results support the idea that biological market dynamics ensure the stable regulation of resource exchange in the evolution of AM symbiosis. However, other evidence suggests that reciprocal regulation represents only a fraction of the forces determining resource exchange in the AM symbiosis, and such reciprocity is only found in a subset of symbionts under specific conditions (195).

The Impact of AM Symbiosis on Aboveground Organs of the Host Plant

The impact of the AM symbiosis goes beyond the root apparatus and involves distal parts of the plant through a fine shaping of the whole plant physiology. The first molecular evidence of a systemic effect was observed at the level of gene expression profiles: changes in transcript pattern, which were not a mere consequence of an improvement in P nutrition, were observed in shoots of *M. truncatula* upon root colonization by AM fungi (196). A transcriptional reprogramming was also reported for other plants such as tomato and maize (198–200). Gerlach and colleagues (200) also performed parallel ionomic and metabolomic analyses showing drastic changes in leaf elemental composition, a general increase in C versus N metabolism, and an accumulation of secondary metabolites. The AM symbiosis therefore influences the physiological status of plant leaves.

The systemic effect of the AM symbiosis was recently shown also to extend to fruits with the potential to increase their nutritional values. Lycopene, carotenoid, and volatile compound contents were significantly increased in fruits of mycorrhizal tomato plants compared to those of nonmycorrhizal plants (201, 202). An overall increase in fruit yield of mycorrhizal tomato plants, as well as qualitative and quantitative changes in amino acid profile, accompanied phenological modifications as an accelerated flowering and fruiting time (203, 204). Not only vegetative but also reproductive traits are therefore under the influence of the AM

symbiotic interaction. This situation has major ecological and agronomical implications.

Such systemic effects have been proposed to depend, at least to some extent, on the action of phytohormones, which are also involved in AM establishment and functioning (205–207). The levels of several hormones such as salicylic acid, jasmonic acid, abscisic acid, auxin, and ethylene are altered during AM colonization (208–211). In addition, salicylic acid, ethylene, and cytokinins are considered negative regulators of fungal penetration and root colonization (205). At later stages, arbuscule development is suppressed by biologically active gibberellins and promoted by DELLA gibberellin repressors (137, 205, 212). By contrast, abscisic acid and auxins positively regulate arbuscule development and functionality (213, 214), while contrasting effects have been described for jasmonates (215).

The alteration of transcriptional profiles and hormonal balance in mycorrhizal plants may also have an impact on the plant response to abiotic and biotic stresses (6, 18, 216–218). AM symbiosis often reduces the damage caused by soil-borne pathogens, while the effect on pests and pathogens attacking from aboveground are more variable and are highly dependent on the combination of AM fungus, plant, and attacker (18). The effect of AM at both the local and systemic scale strongly suggests that the bioprotective role of mycorrhization is not simply related to improved mineral nutrition, changes in the root apparatus, and/or changes in the microbial rhizosphere communities, but rather to the activation of systemic defense responses (218–223). In support of this hypothesis, stress- and defense-related genes are upregulated in mycorrhizal plants, which in turn show increased tolerance to foliar bacterial pathogens (196, 199).

In this context, the combined action of plant hormones and gene regulation may contribute to the generation of a primed status in the plant, allowing a more efficient activation of defense mechanisms in case of a subsequent attack (200). The identification of the full set of defense regulatory elements deployed by mycorrhizal plants and indirectly driven by AM fungi will have important practical implications regarding the effectiveness of the AM symbiosis in biological control and integrated management of pests and diseases.

The Common Mycorrhizal Network

AM fungi can also influence plant community dynamics and plant-plant interactions; this has major implications for natural and agricultural systems. A fascinating feature of AM fungi is the ability of their extraradical mycelium to interconnect individual plants of the same or different species in "common mycorrhizal networks" (CMNs) (224). CMNs are very common in terrestrial ecosystems, where they are thought to play key roles. Plants invest between 10 and 30% of their photosynthetic products in their fungal symbionts and receive in exchange up to 90% of their mineral requirements (4, 192). CMNs represent possible pathways for the movement of soil-derived nutrients and plant-derived carbon within the network and between CMN-interconnected plants. However, the knowledge of how C, N, and P (as well as other nutrients) are exchanged and redistributed via the CMNs is still limited. The transfer of C via CMNs has been demonstrated from autotrophic to achlorophyllous (nonphotosynthetic) plants, yet its transfer between autotrophic plants remains more controversial (225–227). Similarly, the role of CMNs on mineral (e.g., N) transport between plants is not so clear (228).

Moreover, the terms of trade, that is, the relationship between the investment of a given plant into a CMN (amount of assimilated C) and the return of investment in terms of mineral nutrients provided by the CMN, are unresolved. Different cocultivated plants benefit differently from their CMN, depending on the AM fungal species involved (229–231). To address such questions, Walder et al. (232) set up an elegant microcosm experiment with a pair of plants (flax and sorghum) interlinked by a CMN of either *R. intraradices* or *F. mosseae*. Fluxes of C, P, and N were then monitored through C-stable isotope tracing and ^{15}N and ^{33}P labeling. Depending on the fungal species, a strong asymmetry was observed in resource exchange: flax invested little C but obtained up to 94% of the N and P provided by the CMN. Furthermore, the overall biomass was larger when the plants were grown together than in monoculture. Overall, CMNs appear to contribute to the productivity increase that is often observed in intercropping compared with conventional monocropping systems (233). These findings clearly challenge the "biological market" model where the most beneficial partners are favored (14) and suggest that resource exchange in the AM symbiosis is determined by more complex factors (195).

Recently, a new role for CMNs was discovered. Plants can exploit CMNs to transfer defense signals to neighboring individuals. The first demonstration of interplant signaling via CMNs was in tomato plants attacked by the foliar necrotrophic fungus *Alternaria solani*: six defense-related genes were upregulated in uninfected plants that were only connected to the infected individuals by CMN (234). The CMN-mediated transfer of defense signals was also observed between

insect-attacked plants and healthy neighboring plants (235, 236). Altogether, CMN seems to act as a belowground interplant defense communication system. Nevertheless, the nature and the mechanism of signal transfer through the fungal mycelium as well as the ubiquity and ecological impact of interplant signaling in nature remain to be fully understood (237).

CONCLUDING REMARKS

Our understanding of the AM interaction at multiple levels, from cells to ecosystems, is increasingly benefiting from the developments and advances of investigation tools such as "omics" technologies, live cell imaging, stable isotope tracking, and genetic manipulation. Already, genome sequencing and analyses have accelerated our understanding of enigmatic aspects of the genetics and biology of AM fungi. It is likely that mycorrhizal research will rely more and more on multidisciplinary approaches and combinations of analytical techniques—in both controlled and natural conditions—to answer numerous questions concerning the evolution, ecology, and functioning of this fascinating interaction. The holistic knowledge generated by such approaches will be crucial to boost the fruitful application of the AM symbiosis in sustainable agronomical practices to face current challenges in global food production and security.

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Lichenized Fungi and the Evolution of Symbiotic Organization

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INTRODUCTION AND HISTORICAL BACKGROUND

Lichens were generally accepted as an independent group of organisms when Schwendener (1) discovered that they are the result of the association of fungi and algae. This insight was not widely accepted by contemporaries but initiated a scientific revolution as lichens later became the prime example of a mutualistic symbiosis and, indeed, the phenomenon for which the term symbiosis was originally introduced in biology as "symbiotismus" (2, 3). The lichen symbiosis has proved to be one of the most important lifestyles in the *Ascomycota* and is also known from a few *Basidiomycota*. Approximately 20,000 currently known fungal species live as lichens, mostly in species-rich lineages of *Ascomycota* (4). The traditional view of lichens as a mutualistic symbiosis of a fungus and one or several green algae or cyanobacteria has always been debated (5), but it has recently been challenged more than ever by the discovery of numerous additional microorganisms that potentially occur as obligatory participants in the symbiosis.

The self-supporting associations of fungi and their partners usually form a compact and macroscopically recognizable structure, known as the lichen thallus. Different from many other fungi, which reside beneath the surface of their substrates, the lichen thallus is typically developed at the surface of the substrate or completely exposed to the atmospheric conditions, apparently without exploiting the substrates' nutrients (Fig. 1).

THE LICHEN THALLUS

The lichen thallus arose early in evolutionary history with the conquest of land, and the earliest records of internally stratified cyanobacterial and green-algal lichens were from the Lower Devonian (6). These lichens have an unclear relationship to current lichen-forming lineages in the *Ascomycota*, which originated at least as early as the Carboniferous and radiated in the Jurassic and Cretaceous to generate the diversity of the main modern groups (7). The ability to revitalize from dry stages helps the lichen thallus in many species to survive environmental fluctuations with extremes of desiccation and temperatures (8, 9). Lichens are thus prominent at high altitudes and latitudes where conditions become adverse for most other multicellular organisms. Especially in cool habitats, lichen biomass can be substantial, and lichens can then form a dominant part of the vegetation (Fig. 2). By the balance of respiration and photosynthetic energy production in the symbiotic system, many lichen thalli grow slowly but are long-lived. Usually, the fungi dominate the biomass and dictate the shape of lichen thalli. The scientific name of lichens also, by definition, refers to the fungal partner only.

Lichen fungi shelter the algal/cyanobacterial partners beneath a protective peripheral cortex layer (Fig. 3). These layers are developed by the conglutination of fungal hyphae, which seems to be correlated with increased branching and anastomoses formation. The outer walls of the fungi are thickened to form sticky contacts with neighboring hyphae. This appears to be similar to the process generally found with the formation of fungal fruit bodies, but in the case of lichens conglutination and compaction occur in the vegetative mycelium, and this could be one of the key evolutionary innovations to make lichen thallus evolution possible. The conglutination of vegetative hyphae forms a more or less coherent layer by which algal cells can

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Figure 1 Diverse shapes of lichen thalli. (**A**) Coral-like fruticose (shrub-like) thallus of *Cladia retipora*. Photo: Birgitta Strömbäck. (**B**) Foliose (leaf-like) thalli of *Lobaria pulmonaria* and *Lobarina scrobiculata* covering the trunk of a *Salix caprea* in an old-growth spruce-dominated forest. Photo: Mats Wedin. (**C**) Crustose (crust-like) thallus of *Acarospora fuscata* growing on a siliceous rock. Photo: Martin Westberg.

be covered and under which algal populations can form more or less coherent layers within fungal plectenchyma (i.e., tissue-like structure made of fungal hyphae). This developmental process seems to be correlated with massive branching and anastomosing of the hyphae. While genes regulating branching, anastomosing, and conglutination are known from model fungal systems, their presence and regulation in lichenized fungi have so far not been studied. The morphogenetic processes seem to be initiated as soon as the fungi contact appropriate algal cells. Once the thallus morphogenesis is initiated, the algal symbionts can proliferate massively under the fungal protectorate. The mycobionts are triggered by the interaction with the algae to produce diverse, more or less complex thallus morphologies, a wide diversity of lichen-derived secondary metabolites, and fruit bodies for sexual fungal reproduction and/or asexual structures for combined propagation of symbionts. Notably, strikingly convergent thallus morphologies have evolved in several unrelated lineages of lichenized fungi (10). The convergent shapes include complicated morphologies, for example, umbilicate thalli (leafy structures with a central, exclusively fungal holdfast), as well as reductions of complexity



Figure 2 Substantial biomass of lichens. (**A**) Scandinavian costal lichen heath dominated by reindeer lichens (*Cladonia* spp.). Photo: Birgitta Strömbäck. (**B**) Lichen-dominated soil crust community on the Great Alvar of Öland (Sweden). Photo: Martin Westberg. (**C**) Abundant lichen cover dominated by *Pseudocyphellaria homoeophylla* in a New Zealand cool temperate rain forest. Photo: Birgitta Strömbäck.

and reversal to simple crustose lichens or even parasitic lifestyles, or the formation of thalli composed of grain-like fungal-algal propagules.

In certain lineages, there is a strong selectional trend toward vegetative reproduction solutions that disperse the fungal and algal partners. These include soredia, which are granules of varying size that contain algal cells and fungal hyphae, produced in structures of varying size and morphology termed soralia (Fig. 4A). Other types of vegetative propagules are corticate, which form easily detached outgrowths from the lichen thallus, called isidia (Fig. 4B). In several lineages of lichens that reproduce by vegetative means, the production of sexual spore-producing structures is rare or unknown.

While most species of lichenized fungi produce highly organized thalli with internal stratification, several "borderline" lichens produce poorly differentiated

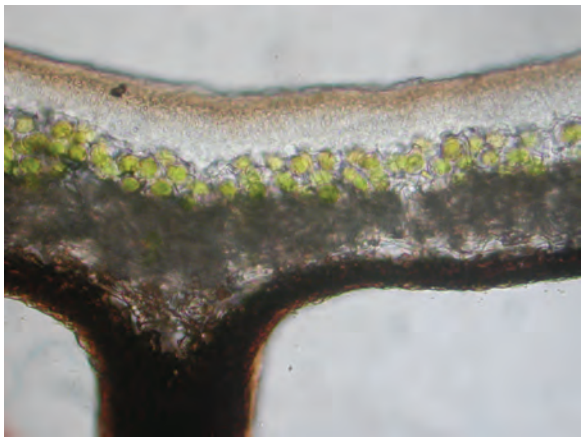


Figure 3 Vertical section through a thallus of *Parmelia saxatilis*, a foliose lichen. A distinct layer of green algal cells is clearly visible under the uppermost cortex; all other structures are made up by fungal hyphae. Photo: Einar Timdal.



Figure 4 Vegetative dispersal in lichens. (**A**) Soralia formed along the thallus margins in *Vulpicida pinastri*. These structures produce soredia, small granules of algal cells surrounded by fungal hyphae. Photo: Einar Timdal. (**B**) Simple, cylindrical isidia formed on the thallus upper surface by *Parmelia saxatilis*. Photo: Einar Timdal.

thalli (11–13). A small number of fungal species also seem to be facultatively lichenized, in the sense that they sometimes are found loosely associated with algae and sometimes are not (14–16). In the *Stictidaceae*, this facultative lichenization is apparently determined by the substrate they grow on, because several species can grow as saprotrophs on dead wood (Fig. 5A) and as loosely lichenized on bark (Fig. 5B). This has been termed "optional lichenization" by Wedin et al. (17). These borderline and optional lichens may be seen as present-day analogs of early thallus evolution, before more advanced forms of thallus organization arose.

THE LICHEN FUNGI AND THEIR PHYLOGENETIC RELATIONSHIPS

The integration of lichenized fungi into the classification of *Fungi* was slow at first because very few lichenologists accepted the consequences of Schwendener's discovery. Vainio (18) was the first to classify at least some higher ranks of the lichen fungi together with other fungi, but the very influential Zahlbruckner (19, 20) treated lichens as a separate group, *Lichenes*, on a level equal to the *Ascomycetes* and the *Basidiomycetes*, which prevented further progress for many years. Nannfeldt (21) argued strongly for integrating lichenized fungi into a natural fungal system, something that was acted upon by Santesson (22–24). Only in the second half of the 20th century was this integrated classification widely accepted by most mycologists, even if the study of lichen fungi nevertheless often is still considered a matter for lichenologists and quite distinct from the study of nonlichenized fungi.

Lichenized lineages are interesting from a phylogenetic-evolutionary perspective, because they primarily have evolved and diverged in the lichenized condition with similar partners. The self-supporting, self-sufficient lichen thallus is likely to be the target of selective pressures very different from the selective forces working on nonlichenized fungi, which creates a substantial difference from what saprobic or parasitic fungi may undergo.

Within the lichens belonging to the *Ascomycota*, microscopic characteristics of the sexual reproductive structures, in particular, fruit body development and organization (25–27), and ascus structures (28–30) helped to refine the taxonomic relationships and were largely the basis of classifications in the late premolecular era. Already at that time, there was a vigorous debate about the number of times that the lichen lifestyle arose in the evolution of fungi. Since it was not feasible to grow axenic fungal cultures, it was only possible to amplify mycobiont genes directly from lichen samples after the development of fungal-specific PCR primers, which excluded the algal partners. With this technical advancement, Gargas et al. (31) were able to provide a first phylogenetic analysis of 18S rRNA genes in lichenized fungi. Their results suggested that the lichen lifestyle arose at least twice in the evolution of ascomycetes. Data from more sequences of lichenized lineages were analyzed by Lutzoni et al. (32) to revisit the fundamental question of lichen origins. The idea of two origins was not rejected, but neither was the possibility of a single origin of lichens in *Ascomycota*. Both studies convincingly showed that the lichen lifestyle was a rare innovation in the evolution of *Ascomycota*, while the latter study pointed out that the symbiotic lifestyle

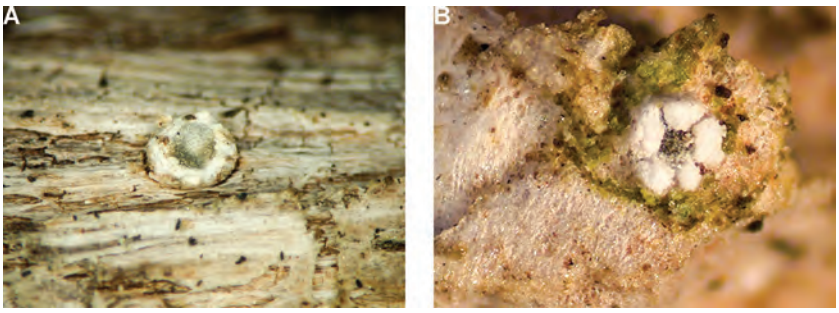


Figure 5 Optional lichenization: *Schizoxylon albescens*. (**A**) Apothecium of a saprotrophic colony on dead aspen (*Populus tremula*) wood. Photo: Lucia Muggia. (**B**) Lichenized morph on aspen bark. Note the green algal colonies around the young apothecium. Photo: Lucia Muggia.

was more frequently lost in major ascomycete groups. Later studies (33) again suggested that lichenization originated multiple times, which seems currently to be the generally agreed upon view.

During the past years our understanding of the phylogeny within the major lineages of lichenized *Ascomycota* increased significantly through a number of investigations involving large numbers of species. An updated classification of *Ascomycota* including the lichenized lineages can be found in Jaklitsch et al. (34), reflecting these discoveries. Extant lichenized fungi occur in at least six of the major lineages within the *Ascomycetes*: the *Arthoniomycetes*, *Coniocybomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Lecanoromycetes*, and *Lichinomycetes* (35–38). One additional lichenized lineage may belong to the *Leotiomycetes*. Different "relaxed clock" scenarios suggested that the origin and diversification of the *Pezizomycotina* occurred in the Cambrian (7). Prieto and Wedin (7) provided information about the timing of the main diversification events in *Ascomycota*, including estimates for classes, orders, and several families of both lichenized and nonlichenized *Ascomycota*, many of which had not been previously dated. The main lineages of lichen-forming *Ascomycota* were shown to have all originated at least as early as the Carboniferous, with successive radiations in the Jurassic and Cretaceous generating the diversity of the main modern groups.

Comparatively few representatives of *Basidiomycota* are lichenized, but recently, the *Cora-Dictyonema* complex was shown to contain a massive number of earlier undistinguished species (39). An investigation focusing on the morphological characteristic evolution in this group showed that the lichenized thallus had evolved progressively from loosely organized filamentous crusts to stereoid and corticioid basidiomata more or less incorporated into the lichenized thallus (40). This indicates that lichenized thalli may have evolved from reproductive structures in their nonlichenized ancestors.

In the overview of lichenized *Ascomycota* below, we select some examples of phylogenetic studies that contributed major advances in our understanding of morphological and chemical characteristic evolution, lichen biology and interactions, and the general understanding of major lineages of each group.

Arthoniomycetes

The *Arthoniomycetes* contain a single order, *Arthoniales*, the second-largest group of lichen-forming fungi, which is a morphologically diverse group of predominantly lichenized representatives, most of which form lichen symbioses together with green algae of the *Trentepohliaceae*. Grube (41) presented a premolecular era overview of taxonomic and phylogenetic concepts for the *Arthoniales*. This account provided discussions of selected morphological characteristics and a key to the genera now placed in the order. This work served as a baseline for subsequent molecular work. Ertz and Tehler (42) presented the first comprehensive phylogeny of *Arthoniales* focusing on lineages previously assigned to *Opegraphaceae* and *Roccellaceae*. Their two-locus phylogenetic study showed that traditional morphological characteristics such as growth form, fruit body type, exciple (a fungal layer of hyphae directly surrounding and derived from the ascoma), hypothecium and ascospore color, ascospore septation pattern, and secondary metabolism are of limited use in delimiting families and genera. They concluded that the high level of phenotypic plasticity might indicate that *Arthoniales* is an old group and that phenotypically characterized genera are paraphyletic. Frisch et al.'s recent analysis (43) focused on the heteromorphic family *Arthoniaceae* and demonstrated that previous classifications of this family did not reflect evolutionary

patterns. According to the phylogenetic hypothesis, lichen secondary metabolites such as pulvinic acid derivates and red pigments do not characterize monophyletic groups above the genus level. The study also revealed that the lichen-parasitic lifestyle in *Arthoniaceae* has evolved more than once and is found in four independent lineages of the *Arthoniaceae* clade and in the *Bryostigma* clade. *Arthoniomycetes* strains with chlorococcoid photobionts are restricted to the *Bryostigma* clade and *Chrysotrichaceae*, while the only saprophytic *Arthonia* species in the phylogeny is related to *Arthonia radiata* and grouped with lichenized taxa. The phylogenetic data provide a coherent framework for delineating further monophyletic groups in *Arthoniaceae* in the future.

Coniocybomycetes

Coniocybomycetes is a small group (one order and family, currently two genera with ca. 30 species) of crustose lichens with prototunicate (thin-walled and evanescent) asci and mazaedia-producing (mazaedium: an accumulation of loose, maturing spores covering the ascoma surface), stalked ascomata. This is the most recently described class of *Ascomycota* and is apparently related to the *Lichinomycetes* (38), but this relationship is not supported by any obvious morphological characteristics. *Coniocybomycetes* is one of the few lichen groups from which a fossil is known, which Prieto and Wedin (7) utilized in their dating of the major groups of *Ascomycota*.

Dothideomycetes

The *Dothideomycetes* is a very large fungal group characterized by ascolocular ascoma development, and only some representatives are lichenized. Schoch et al. (44) presented a comprehensive phylogenetic study of representatives of 41 families using sequences from five genes. Notably, the ancestral reconstruction of basic nutritional modes suggests numerous transitions from saprobic life histories to either plant-associated or lichenized modes. Nelsen et al. (37) integrated the primarily lichen-forming families *Trypetheliaceae*, *Monoblastiaceae*, and *Arthopyreniaceae* in a phylogeny of *Dothideomycetes*. Pérez-Ortega et al. (13) introduced the new order *Collemopsidiales*, which contains *Xanthopyreniaceae*, an interesting family including borderline lichens, many of which occur in rocky intertidal habitats, as well as lichenicolous (lichen-inhabiting) fungi.

Eurotiomycetes

Eurotiomycetes is a very large and morphologically heterogenous group in which only some representatives are lichenized, all within the subclass *Chaetothyriomycetidae*, which is an assemblage of ecologically diverse species ranging from mutualistic lichenized fungi to human opportunistic pathogens. A multigene phylogenetic study of rock-dwelling fungi concentrating on the *Verrucariales* suggested that this lichenized order is one of the ascomycete groups in which lichenization has independently evolved on a hostile rock surface (45). The delimitation of orders and families in *Chaetothyriomycetidae* was reassessed by Gueidan et al. (35) in a broad phylogenetic study which classified four orders and 10 families in *Chaetothyriomycetidae*.

Lecanoromycetes

This class comprises the largest group of lichenized fungi and one of the most species-rich classes in the kingdom of *Fungi*. Traditional concepts of classification included reproductive structures, in particular, of ascomata and ascus apex structure (which often contain characteristic amyloid features). These traditional concepts were challenged when comprehensive sequence sampling of taxa was available (46–48). Miadlikowska et al. (49) demonstrated that the *Acarosporomycetidae* and *Ostropomycetidae* are monophyletic subclasses, whereas the delimitation of the largest subclass, the *Lecanoromycetidae*, remained uncertain. This and the previous phylogenies confirmed that ascus apex morphology cannot be consistently used as a cardinal characteristic for family-level classification of lichen-forming fungi (50).

One important approach in understanding the evolution of phenetic features of lichens is to study large genera, which are pragmatically circumscribed by key characteristics. In many studies these key characteristics proved to be misleading and, rather, were symptoms of massively parallel evolution and thus produced by unrelated fungi. Examples of such misleading key characteristics are prototunicate asci and mazaedia, which used to characterize the old order *Caliciales* (including pin- and stubble-lichens), a group that in its old circumscription now has been shown to be extremely polyphyletic (38, 51) and where the families *Caliciaceae* and *Sphaerophoraceae* are now classified in the *Lecanoromycetes* (31, 38). Another example of this phenomenon was provided by Schmull et al.'s (52) analysis of the heterogenous *Lecidea* (containing 1,200 species), which are usually characterized by black fruit bodies on a crust-like thalli and single-celled, hyaline ascospores. As expected, the analyzed species of *Lecidea sensu lato* and putatively related taxa were scattered within *Lecanoromycetidae*, and some were even placed outside currently recognized orders in *Lecanoromycetidae*.

Most of the lichen fungi that form symbioses with filamentous *Nostoc* cyanobacteria belong to the *Lecanoromycetes*. *Nostoc* has a sheath around the filamentous thread-like colonies that swells and becomes extremely gelatinous when wet, and this gives many cyanolichens a gelatinous appearance when moist. Within the *Lecanoromycetes*, most gelatinous lichens are classified in the *Collemataceae*. This is yet another example of how classifications have been based on misleading key characteristics, because a number of gelatinous, *Nostoc*-containing groups formerly placed in *Collemataceae* are nested within another *Peltigerales* family, the *Pannariaceae* (53, 54). Symbioses with *Nostoc* have clearly arisen several times. Depending on the reconstruction method, most or all transformations in thallus structure within *Peltigerales* took place from a nongelatinous to a gelatinous, *Nostoc*-containing thallus (53).

Within *Lecanorales*, the phylogeny of the largest lichen family, *Parmeliaceae*, was studied in a number of papers; for example, Crespo et al. (55) showed that morphological characteristics discriminated the main clades well but that the interpretation of the morphological diversity had been quite superficial. The morphological diversity was indeed found to be substantial in this family when Divakar et al. (56) showed that the lichenicolous lifestyle originated independently three times within *Parmeliaceae* ca. 24 million years ago.

Gaya et al. (57) studied the *Teloschistales* using a supermatrix approach and showed that a progressive, cumulative addition of taxa to the matrix analyzed with a resulting increasing amount of missing data did not affect the support and resolution much but that the monophyly of the order was inconsistent depending on the combination of loci and taxa. In the *Teloschistaceae*, Arup et al. (58) proposed a completely reorganized generic classification and investigated how the apparently large morphological plasticity affected the characterization of genera and species. Secondary metabolites were frequently found to better serve as characterizing traits than morphology, at least in parts of the family.

Within *Ostropomycetidae* Baloch et al. (59) suggested that the evolution of lifestyles and ascomatal morphologies in this group was very plastic, as shown by the multiple evolution of perithecioid ascomata in the *Gyalectaceae*. Early evolutionary splits in the *Ostropomycetidae*, the second-most species-rich subclass of lichenized *Ascomycota*, were considered by Resl et al. (60) in their analysis of trapelioid fungi. The uncertain phylogenetic resolution of the c. 170 species prevented a clear backbone concept for the subclass. A monophyletic group of nine core trapelioid genera was found, which do not form the sister group of *Ostropales*, but the hypothesis of a sister group relationship of trapelioid genera and *Baeomycetaceae* or *Hymeneliaceae* could not be rejected. Recently, Miadlikowska et al. (61) provided the most comprehensive phylogenetic survey of the entire *Lecanoromycetes* class using a multigene maximum likelihood analysis with a cumulative supermatrix approach. However, the analysis of this massive data set (1,139 taxa) revealed that the cumulative addition of taxa with an increasing amount of missing data leads to relatively stable representation of relationships for many families and orders, but also in substantial loss of phylogenetic resolving power and support for deep phylogenetic relationships.

In the *Graphidaceae*, a very large family of >2,000 species, Lumbsch et al. (62) studied character evolution and found that certain characteristics (secondary metabolites, in particular) had a high frequency of reversible phenotypic state changes, whereas others, such as photobiont, hymenial persistence or ascoma aggregation, exhibited low frequency of transformations. But even in the characteristic with the highest number of state changes (changes in secondary metabolite composition), the shifts were highly structured phylogenetically, suggesting that the evolution of the characteristic, rather than the character state itself, can be used to predict phylogenetic relationships with certain accuracy.

Leotiomycetes

Trizodia acrobia is a borderline lichen and the sister group of *Leotiomycetes*. The lineage was discovered by Stenroos et al. (63) in a phylogenetic analysis of bryophilous ascomycetes. *Trizodia* is ecologically unique in its association with cyanobacterial colonies (mostly *Nostoc*) growing on the tips of peat mosses (*Sphagnum* spp.). *Trizodia* was consistently present in all *Sphagnum-Nostoc* associations studied. It envelops the cyanobacterial colonies both on the moss surface as well as inside the leaf but does not form organized thallus structures.

THE ADVANCING GENOMICS PERSPECTIVE FOR UNDERSTANDING LICHEN SYMBIOSES

Increasing the number of loci used to determine phylogenetic relationships is now possible through characterization of entire genomes of lichens. Genome-scale datasets may lead to the development of consistent, well-supported hypotheses about the evolution of lichenized fungi. A first step toward a phylogenomic analysis of lichen-forming fungi was undertaken in an exem-

plary phylogenomic study of the genus *Rhizoplaca* (*Lecanoromycetes*) by Leavitt et al. (64).

Current high-throughput sequencing technology has opened new opportunities for studying organisms that grow slowly and are difficult to establish and grow in axenic cultures. These difficulties explain why comparative genomics of lichen fungi lags behind the advances made so far in other fungal groups. Meanwhile, genetic manipulation, such as transformation, of lichens has been attempted (65). Numerous lichen genome projects have been started in the past years, and some preliminary data are already available. Basically, two strategies are followed in lichen genomics. The more traditional approach is the acquisition of genetic information from the individually cultured symbiotic partners. With the alternative metagenomics approach the total symbiotic association is first sequenced and the genetic information is then assigned to symbiotic partners by bioinformatics analysis. Such progress is possible by exploiting technologies that have significantly improved the quality of sequence assemblies (e.g., by "mate-pair" sequencing) and by powerful bioinformatics pipelines. Apart from gaining information on basic genomic features such as genome size and predicted numbers of genes (Table 1), several studies provided more detailed analyses of interesting functions.

Some of these analyses focused on the biosynthetic pathways of secondary metabolites of lichenized fungi (including depsides and depsidones, as classes of coupled phenol carboxylic acids). Sequencing the 34-Mb genome of the *Cladonia grayi* mycobiont revealed new insights in the production of the lichen depsidone grayanic acid (66) and suggested that a single polyketide synthase synthesizes two aromatic rings on tandem acyl carrier proteins and links them into a depside and that the transition from depside to depsidone requires only a cytochrome P-450 mono-oxygenase. Sequencing of the *Cladonia uncialis* mycobiont revealed a putative biosynthetic gene cluster leading to usnic acid, a dibenzofuran derivative (67). Although no typically crystallized lichen substances have been recorded for *Peltigera membranacea*, a large number of mycobiont and photobiont genes and gene clusters associated with secondary metabolite biosynthetic pathways have been identified in its metagenome, and an unusual trans-AT polyketide biosynthetic pathway of a type known only from other bacterial-eukaryote symbioses has been identified in the *Nostoc* photobiont (68).

In addition, the genome sequencing provided evidence that the tight association of fungi and prokaryotes might have favored horizontal gene transfer events. One such event was detected in the methylammonium permease family between prokaryotes and the *C. grayi* mycobiont (69). Subsequently, McDonald et al. (70) suggested that lichen-forming fungi are losing this gene family at a slower rate than other fungal lineages.

The integration of further available (meta)omics approaches, such as (meta)transcriptomics or (meta) proteomics, provides new and complementary insights into lichen symbiosis. In this context, transcriptomics reveals which of the total set of genes are activated under certain conditions, whereas proteomics suggests

Table 1 Lichenized fungal species for which genome information is available (as of March 2016)

Species	Class	Genome size (Mb)	Gene no.	Reference
Acarospora strigata	Lecanoromycetes	27	?	70
Arthonia rubrocincta	Arthoniomycetes	26	?	70
Cladonia rangiferina	Lecanoromycetes	32	9,211	122
Cladonia grayi	Lecanoromycetes	34	?	http://genome.jgi.doe.gov/Clagr2/Clagr2.home.html
Cladonia macilenta	Lecanoromycetes	37.1	7,322	123
Cladonia metacorallifera	Lecanoromycetes	36.6	11,361	124
Cladonia uncialis	Lecanoromycetes	30	?	67
Dibaeis baeomyces	Lecanoromycetes	35	?	70
Endocarpon pallidulum	Lecanoromycetes	41	?	70
Endocarpon pusillum	Lecanoromycetes	37.5	9,285	125
Graphis scripta	Lecanoromycetes	36	?	70
Gyalolechia flavorubescens	Lecanoromycetes	34.4	9,695	126
Leptogium austroamericanum	Lecanoromycetes	46	?	70
Peltula cylindrica	Lecanoromycetes	32	?	70
Physcia stellaris	Lecanoromycetes	59	?	70
Umbilicaria muehlenbergii	Lecanoromycetes	34.8	8,294	127
Xanthoria parietina	Lecanoromycetes	32	10,800	http://genome.jgi.doe.gov/Xanpa1/Xanpa1.home.html

which functions are actually translated into protein functions. Wang et al. (71) provided an initial full-length cDNA library as a reflection of the transcriptome, using the cultured mycobiont of the desert lichen *Endocarpon pusillum*. However, because a symbiotic context is missing, the significance of the detected gene expression for symbiosis remained unclear. In a subsequent study of the same lichen, Wang et al. (72) also analyzed dehydrated thalli and confirmed expression of 23 candidate stress responsive genes, selected from a larger set found with mycobiont cultures exposed to PEG-induced drought stress. There is also ongoing RNA-Seq work aiming to compare gene expression in the mycobiont alone in pure culture with the mycobiont in the symbiotic state to identify genes that are differentially expressed and might therefore be correlated with symbiotic interactions. Gene expression studies elucidate relative levels of active genes (73). Also, epigenetic modifications such as the presence of 5-methylcytosine in lichen genomes can be determined in conjunction with next-generation sequencing platforms.

Moving a step further, Junttila and Rudd (74) used high-throughput next-generation sequencing and expressed sequence tag sequence data to present a first transcriptome of the eukaryotic partners in the thalli of the reindeer lichen *Cladonia rangiferina* (with 62.8% reads of fungal and 37.2% of algal origin). Even though a higher percentage of algal reads was found in the wetted thalli, gene ontology terms (http://geneontology.org/) and identified Kyoto Encyclopedia of Genes and Genomes pathways (www.genome.jp/kegg/) largely agreed with eukaryotic metaproteome patterns found by Schneider et al. (75). Junttila et al. (76) tracked the expression profiles during desiccation and rehydration using microarray analyses, but the data do not provide detailed insights into the regulatory processes. Most of the differentially expressed genes do not show sequence similarity to known genes. It is, however, remarkable that the largest changes of gene expression are observed only minutes after rehydration.

Given the complexity of lichens, it is not surprising that the functional contributions of genes are organ-specific and modified by pertinent ecological and developmental conditions. The partially annotated *P. membranacea* metagenome revealed the presence of mycobiont genes encoding galectin-like proteins, which are a family of proteins defined by their binding specificity for β-galactoside sugars (77). RNA-Seq data further showed that one of these genes, *lec-1*, was differentially expressed in rhizines, a purely fungal tissue, when compared to the remainder thallus, composed of both mycobiont and photobiont (73).

While forthcoming comparative genomics studies will inform us about the evolutionary dynamics of lineages diverging in lichen-symbiotic stages, transcriptomics, proteomics, and metabolomics will improve our understanding of the symbiotic regulation and processing. *Lobaria pulmonaria*, a tripartite lichen (including both a green algae and a cyanobacterium) widely distributed in the Northern hemisphere, has been featured in publications that have explored metaproteomic issues (75, 78). Most algal proteins were assigned to energy production and conversion. Carbohydrate transport and metabolism were significant in both eukaryotic partners, but fungal functions were more diverse, with substantial read numbers suggesting biogenesis and posttranslational modification. The bacterial fraction (see also below) in the metaproteome was dominated by proteins from *Alphaproteobacteria*. The identified proteins are primarily involved in energy conversion and carbohydrate metabolism, as well as responses to stress. Also, some of the bacterial proteome spectra suggested a role of bacteria in secondary metabolite synthesis, but this has not been resolved in full detail so far.

FUNGAL-ALGAL CONNECTIONS AND INTERACTIONS IN THE THALLUS

The main role of the algal partners is the provision of photosynthetically fixed carbohydrates as the energetic basis for the self-sustained lifestyle. The lichen-forming fungi take up osmotically active monomeric sugars from the algae, which are then further metabolized. Typically, green algae supply polyols (ribitol in chlorococcal algae and erythritol in filamentous *Trentepohliales*), whereas glucose is provided by cyanobacterial algae. Apparently the process of lichenization also stimulates upregulation of photo-protective mechanisms in the photobiont (79). In turn, the photobiont stimulates the antioxidant system of the mycobiont (8, 9). Currently, it is not well known which chemical signals are transferred during the initiation and onset of lichenized stages or what effects they have in the symbiosis. By comparing algal strains, Meessen and Ott (80) detected characteristic metabolites in lichen-forming algae, such as the cyclic dipeptides cyclo-L-leucyl-L-tyrosine and cyclo-(L-tryptophyl-L-tryptophyl), rhamnose, and indole-3-carbaldehyde, a precursor or a degradation product of the phytohormone indole-3-acetic-acid. Because hyphal branching is not stimulated with these substances alone, but when unicellular algae grow in proximity (81), further metabolites seem to be involved in the signaling of lichenization. Such molecules probably include the

phytohormones abscisic acid and ethylene, as well as others (82, 83, 84).

The fungal partner frequently produces attachment or penetration structures (appressoria or haustoria, respectively) of different kinds at the mycobiont-photobiont interface. Water and dissolved nutrients can move readily between the bionts at the contact zone, within a hydrophobic coat that the fungus produces over the algal cell surface (85, 86).

Lichen fungi associate with only about 120 known species of algae, most of them green algae, and a few cyanobacterial algae (86), although the species taxonomy is so poorly understood in lichenized cyanobacteria that these currently cannot be identified to the species level (87). The photosynthetic partners are green algae in about 90% of lichen species. Hence, 10% of the lichens take advantage of bacterial nitrogen fixation and associate with cyanobacterial algae, usually with filamentous *Nostoc* strains. Another strategy is the additional association with *Nostoc* strains in specialized organs (cephalodia; Fig. 6A) in or on a green-algal thallus (88), resulting in tripartite lichens, which apparently evolved as a segregation of functions. The higher proportion of heterocysts suggests a main role of cephalodia in nitrogen fixation (89). On the other hand, several cyanobacterial lichens may also have green algae in addition to the cyanobacteria in their photosynthethic layer (90), which might widen their ecological amplitude in cool habitats. Few fungal species are even able to use either eukaryotic green or prokaryotic blue-green algae to develop independent symbiotic thalli (91). These phycosymbiodemes, primarily found in the order *Peltigerales* of the class *Lecanoromycetes*, either have similar morphologies or are shaped profoundly differently (Fig. 6B). When growing separately, they were described in independent genera, which has blurred the understanding of their phylogenetic relationships (92).

With very few exceptions, the green algae propagate clonally and do not form sexual structures in the lichenized stage. The apparent suppression of the sexual capacity of the algal partner by the lichen-forming fungi could be interpreted as a selfish strategy to avoid genetic diversification of the partner and to maintain efficient control over the algal physiology.

Refined molecular analyses meanwhile have helped to improve our knowledge about lichen photobiont diversity—their phylogenetic relationship as well as their patterns of association with their fungal hosts. Major mycobiont lineages seem to vary by their overall spectra of preferences for algal groups. While the majority of *Arthoniomycetes* have a preference for *Trentepohliaceae* (except for some species assigned to *Arthonia*), *Lecanoromycetes*, particularly members of the class *Chaetothyriomycetes* (above all, *Verrucariales*), associate with a broader range of green algae (43, 61, 93).

Recent work suggests that species of lichenized fungi frequently vary in their algal specificity and selectivity. This correlates with the range of the species and climatic differences; i.e., the same lichens occurring in different habitats often associate with related algal species with different ecological preferences (94, 95). This flexibility supports the hypothesis of habitat-adapted symbioses proposed by Rodriguez et al. (96), which suggests that the environment determines the optimal partnership of symbionts. Wedin et al. (97) showed that *Diploschistes muscorum* has an even more flexible photobiont strategy than earlier believed. *Diploschistes* starts as a parasitic fungus infecting the unrelated lichen *Cladonia* (Fig. 7), eventually taking over and forming an independent thallus. Although the *Cladonia*

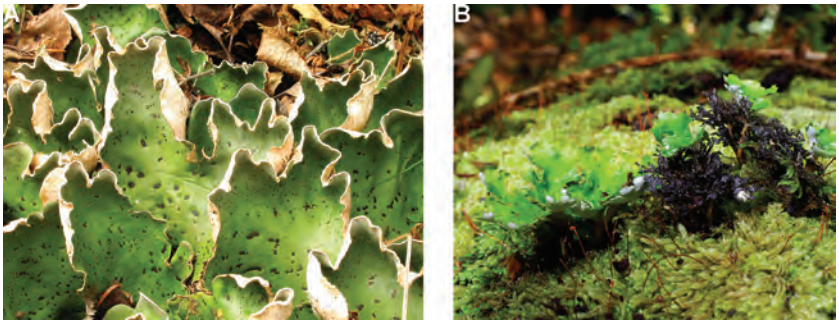


Figure 6 (**A**) Cephalodia visible as dark structures that include cyanobacteria, on *Peltigera aphtosa*. Photo: Einar Timdal. (**B**) Phycosymbiodemes with different morphologies: *Sticta* with joined photomorphs. Note green-algal foliose parts growing out from the basal cyanobacterial fruticose parts. Photo: Mats Wedin.



Figure 7 Symbiotic invasion. The transition of *Cladonia* thallus into *Diploschistes* thallus by invasion of the latter. (**A**) Uninfected *Cladonia symphycarpa* thallus. Photo: Einar Timdal. (**B**) *Cladonia* thallus with clear *Diploschistes* infection (whitish areas) with typical *Diploschistes* apothecia (dark patches in the whitish areas). Photo: Einar Timdal. (**C**) An almost complete takeover by *Diploschistes*, with only small remnants of *Cladonia*. Photo: Martin Westberg.

used the same photobiont at all investigated sites, *Diploschistes* associated with different photobionts at all three sites, keeping the original *Cladonia* alga at one site but replacing it with others in the two other sites (97). This suggests a very generalistic photobiont strategy in this lichenicolous lichen.

Rikkinen et al. (98) suggested that lichen fungi in the *Peltigerales* form ecological photobiont guilds, within which the fungi share related photobiont cyanobacteria. Such associations can be seen both among an ecological assemblage of epiphytic macrolichens, the *Nephroma* guild, and among a group of predominantly terricolous cyanolichens, the *Peltigera* guild, where the photobionts with each guild are closely related *Nostoc* strains. Several authors have likewise observed similar photobiont-mediated guilds in green-algal lichens (5, 99).

ADDITIONAL FUNGI IN THE LICHEN SYMBIOSIS

Because lichen thalli are persistent and vary morphologically, they provide a rich diversity of small-scale niches for various microorganisms, in particular fungi and bacteria (discussed below). Lichenicolous fungi comprise all fungal species living in or on lichens, apart from the thallus-forming fungus itself (the mycobiont). This biological group has been studied extensively and was recognized even before the symbiotic nature of their lichen hosts was established. More than 1,800 species of lichenicolous fungi have been described (100), but as numerous new species are still being discovered, their precise number is unknown and is clearly currently much underestimated. The evolutionary origin of the lichenicolous lifestyle is diverse, but a substantial number of lichenicolous fungi apparently evolved after delichenization of originally lichenized lineages. Because most lichenicolous fungi seem to exploit their hosts without rapid destruction, they are highly specific for their hosts and for particular symbionts of the hosts.

Typically, lichenicolous fungi are recognizable by conspicuous reproductive structures or by their symptoms such as discolorations or gall-like hypertrophications. Some other species associate with lichens but remain cryptic. Isolation techniques and axenic cultivation revealed that these species either reside on the surfaces of the thalli (101–103) or occur internally in lichen host thalli (also known as “endolichenic fungi”) (104). Given the abundance and the potential of culturable endolichenic fungi to produce secondary metabolites, it might be debated whether these fungi have a biological effect on their host or influence the pheno-

type in various ways. However, even though microscopic evidence already demonstrates the endolichenic growth of fungi besides the mycobiont in lichens (86), it is not clear which of these fungi grow in lichens or reside as spores or otherwise in dormant stages.

Culture techniques have retrieved a surprisingly large number of fungal species from lichens (105), but these numbers represent only a subset of the total fungal diversity present in environmental samples. Therefore, culture-independent methods are now also employed to characterize the mycobiome of lichen thalli. Using DNA-fingerprinting techniques, Fleischhacker et al. (106) found a high diversity of lichen-associated fungi without correlation with the presence of externally visible lichenicolous fungi. Zhang et al. (107) provided an overview of diversity and distribution of fungi in lichens from an Arctic habitat using next-generation sequencing. Their study of the lichen mycobiome indicated that lichens harbor fungi related to those with diverse ecological contexts. Lichens thus represent a reservoir or an evolutionary hotbed for fungi that may have a role in other habitats, but the specific conditions in fungi with recurrent and prolonged cryptobiotic stages hardly favor the rise of, for example, a biotrophic plant pathogen. Studies in *Lobaria* revealed the high specificity of *Tremella lobariacearum* (108), but it is not known whether the same is true for nonsymptomatic occurrences of *Tremellomycetes*. Refined microscopic techniques can reveal to what extent the scattered occurrence of tremellalean fungi might influence the morphology of the host. Millanes et al. (109) described *Cystobasidiomycetes* as a new lineage of *Pucciniomycotina*. The lichenicolous members of this lineage, which primarily comprises yeasts, cause conspicuous fertile hypertrophications of their hosts. Recently, this lineage was found frequently in *Parmeliaceae*, and on the basis of their occurrence in the upper cortex it has been suggested that these yeasts play a role in the formation of the upper cortex (128). This exciting hypothesis needs further testing and confirmation.

BACTERIAL PARTICIPATION IN LICHEN THALLI

Fungi associated with lichens have been studied extensively for a long time, but the ubiquitous presence of bacteria in lichen thalli received more attention only comparatively recently. Sequencing of lichen-associated bacterial communities revealed information about their diversity on lichen thalli (110–118). *Alphaproteobacteria* usually dominate the bacterial communities in lichens, while other bacterial groups (frequently *Acidobacteria*, *Actinobacteria*, and *Betaproteobacteria*) are also found in significant numbers. One group of *Alphaproteobacteria*, the lichen-associated *Rhizobiales*, is a clade of bacteria so far known only from lichens (113, 115). Sequence-based data are complemented with microscopic information, in particular, employing fluorescence *in situ* hybridization (119). The distribution of bacteria belonging to certain groups can be visualized under the microscope using specific probes (confocal laser scanning microscopy). Bacteria often form biofilm-like communities on the lichen thalli and are usually tightly connected with the fungal structures, in particular, with hydrophilic surfaces of the lichens. The bacteria can also intrude to various depths in the intercellular matrix of the upper cortex and are occasionally also found inside the hyphae of the fungal hosts (120). The lichen-associated bacterial communities so far investigated are host-specific in their composition (112, 115). A clear shift in microbiome profile is observed when the parasitic lichen *D. muscorum* infects, and eventually overcomes, *Cladonia symphycarpa* to form a thallus of its own (97) (Fig. 7). Here, the *Alphaproteobacteria* population dominating the *Cladonia* microbial community decreases during the transition, and the numbers of *Betaproteobacteria* or *Chloroflexi* increase substantially when *Diploschistes* takes over, supporting the view that the microbial community is highly species-specific in lichen thalli. Multiomics approaches to the bacterial communities of lichens finally suggest potential contribution to a number of symbiotic functions in lichens (78).

CONCLUSIONS

Phylogenetic studies significantly improved our understanding of the evolution and phenotypic diversification of lichenized fungi and their partners. Lichen thalli are dominated by the primary fungal symbiont, which controls the characteristic photosynthetic partners. In addition to these commonly known symbionts, unknown numbers of additional organisms can participate in the symbiont system (121). The analysis of massive amounts of molecular data from lichens, which has just started to be collected, will help us to further understand the role of these complex and fascinating partnerships.

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Fungal Plant Pathogenesis Mediated by Effectors

37

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INTRODUCTION

The interactions between fungi and plants encompass a spectrum of ecologies ranging from saprotrophy (growth on dead plant material) through pathogenesis (growth of the fungus accompanied by disease on the plant) to symbiosis (growth of the fungus with growth enhancement of the plant). We consider pathogenesis in this article and the key roles played by a range of pathogen-encoded molecules that have collectively become known as effectors.

Pathogenesis can be defined in terms of either the pathogen or the host. In the former the key factor is reproduction and propagation of the fungus while having a negative impact on the growth rate or product quality of the susceptible host plant. In the latter we focus on the expression of symptoms induced by the pathogen, which range from visible sporulation of the pathogen on an otherwise apparently healthy plant, as in the case of biotrophic pathogens such as powdery mildews and rusts, to the spots, blotches, and rots caused by necrotrophic pathogens. In all cases there is diminution of the reproductive potential of the host, and from an agricultural perspective, reduced quantity and or quality of the desired plant product.

Phytopathogenesis is also caused by oomycetes, bacteria, and viruses and even by animals such as nematodes. Theories of pathogenesis have been developed collectively between these pathogen types, but in this article we consider only the fungi and focus on a small number of key diseases that have formed the bulk of the experimental models.

Much progress has been made in the past decade in our understanding of the mechanisms of infection of plants by these pathogens with different lifestyles, and the key unifying concept is the fungal effector. Effectors are defined as pathogen molecules that have an impact on the outcome of the interaction with the host. This usage was adopted from the medical literature (1), initially in studies of plant pathogenic bacteria (2). The concept has also been adopted by the plant-fungal research community and encompasses effectors that have both positive and negative impacts on pathogen proliferation (3, 4). Here, we mainly focus on effectors whose intrinsic functions and host targets are known.

All plants are resistant to the majority of fungal species. Plants possess PRRs that recognize MAMPs (also known as pathogen-associated molecular patterns) and mediate MTI (or pathogen-associated molecular pattern-triggered immunity), an array of basal defense responses effective against potential pathogens (5). Successful pathogens secrete effectors that suppress MTI and alter host plant physiology to their own advantage (6). In turn, plants have evolved immune receptors, also known as resistance proteins, which recognize effectors, resulting in ETI (7). ETI usually involves the HR that induces localized cell death and is normally sufficient to combat biotrophic plant pathogens that feed only on living cells. Hemibiotrophic pathogens start infection biotrophically, but at the end of the infection cycle when host tissues collapse, they start to feed as a necrotroph on dead host tissue. Yet other pathogens, known as the necrotrophs, were considered to be primitive pathogens that simply kill host tissue before they start to feed on it. However, more detailed analysis of the infection process and comparison of genome sequences has shown that these pathogens use effectors to subvert the resistance process to create appropriate conditions for colonization, a process called NETS (8–10).

Abbreviations

CC, coiled coil; EDS, enhanced disease susceptibility pathway; ETI, effector-triggered immunity; HGT, hori-

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zontal gene transfer; HR, hypersensitive response; HST, host-selective toxins; LRR, leucine-rich repeat; MAMP, microbe-associated molecular pattern; MTI, MAMP-triggered immunity; NBS, nucleotide binding site; NE, necrotrophic effector; NEP1, necrosis- and ethylene-inducing protein 1; NETS, necrotrophic effector-triggered susceptibility; NIP1, necrosis-inducing protein 1; NLP, necrosis- and ethylene-inducing protein 1-like protein; PCD, programmed cell death; PR, pathogenesis related; PRR, pattern recognition receptor; RIP, repeat-induced point; RLK, receptor-like kinase; RLP, receptor-like protein; ROS, reactive oxygen species; sRNA, small RNA; SSCP, small secreted cysteine-rich protein; TIR, toll-interleukin receptor.

ENTRY AND FEEDING OF FUNGAL PATHOGENS

Pathogenic fungi enter plants via natural openings (e.g., stomata) or wounds or penetrate directly using a penetration peg produced by an appressorium (Fig. 1). The key attribute of biotrophic pathogens is the haustorium, a specialized feeding organ for the retrieval of nutrients. Haustoria develop by local penetration of the cell wall and invagination of the plasma membrane, which is surrounded by an extrahaustorial matrix through which nutrient uptake and communication with the host take place (11). In powdery mildews, haustoria are formed directly from the appressoria in epidermal cells. The rusts and smuts invade through stomata and then produce an intercellular mycelium that surrounds mesophyll cells from which a haustorium mother cell and subsequently a haustorium is produced. Hemibiotrophic pathogens encompass a range of pathogenic strategies. Some, such as *Colletotrichum* spp. and *Magnaporthe oryzae*, produce intracellular feeding structures known as biotrophic interfacial complexes (12), while others, such as *Cladosporium fulvum* and *Zymoseptoria tritici*, remain extracellular within the apoplast throughout their lifecycle (13). Vascular wilt pathogens colonize xylem vessels and are in close contact with parenchyma cells.

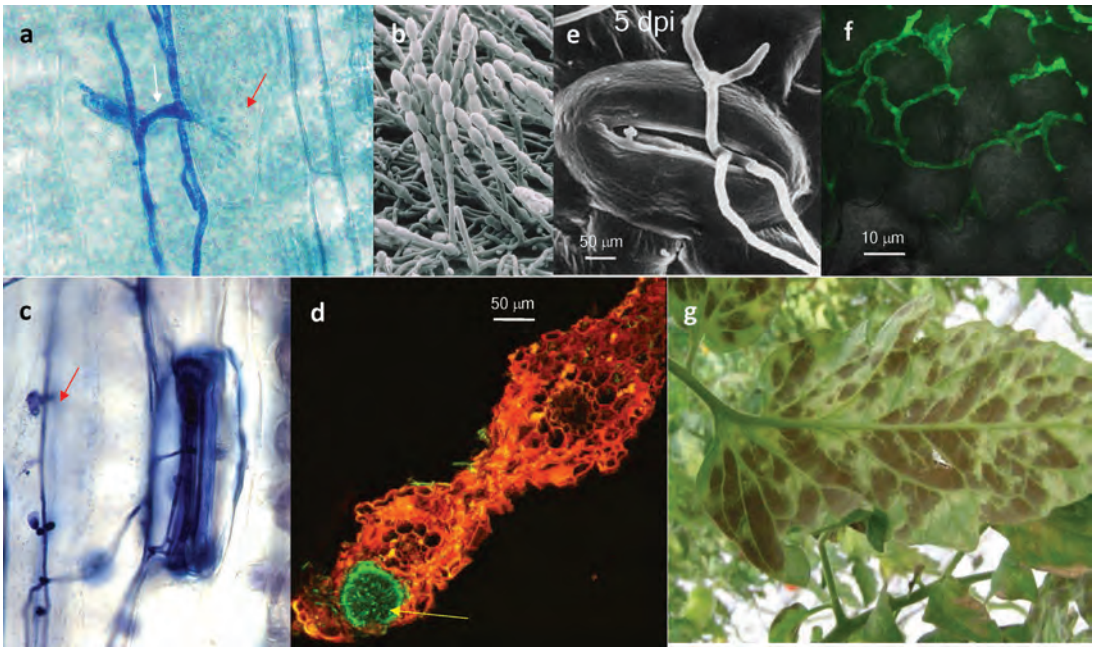


Figure 1 Typical penetration, feeding, and reproductive structures associated with three contrasting fungal pathogens. (**a,b**) Barley powdery mildew, *Blumeria graminis* f.sp. *hordei* (courtesy C. Ge). (**a**) Epiphytic hyphae penetrating through the epidermis and the finger-like haustoria in the epidermal cell. (**b**) Conidiophores bearing abundant conidia. (**c,d**) Wheat septoria nodorum blotch, *Zymoseptoria tritici* (courtesy K. Rybak). (**c**) Epiphytic hyphae penetrating via hyphopodia (arrows) or through stomata. (**d**) A green fluorescent protein-expressing strain under epifluorescence. The arrow shows a pycnidium containing abundant pycnidia. (**e,f,g**) Tomato leaf mold, *Cladosporium fulvum* (Courtesy JC and PdW). (**e**) Penetration of a stoma by adventitious (runner) hyphae. (**f**) Growth of hyphae around tomato mesophyll cells. (**g**) Conidiophores bearing abundant conidia on the underside of an otherwise healthy tomato leaflet.

FUNGAL INFECTION STRATEGIES

The infection strategies employed by fungal plant pathogens depend on where they thrive in their host plants. The cell wall is a major physical and chemical barrier obstacle because it contains the plant waxy cuticle, (lignified) cell walls, antimicrobial metabolites, and PR proteins (14). The sequencing of many fungal genomes has shown that pathogenic fungi contain many genes encoding different classes of cell-wall-degrading enzymes. The expression of these genes is highly upregulated during infection by necrotrophic plant pathogens (15). Biotrophic pathogens generally contain fewer genes encoding cell-wall-degrading enzymes than hemibiotrophs and necrotrophs (6). Necrotrophic fungi often also produce secondary metabolites that are toxic to plant cells, thereby facilitating their necrotrophic lifestyle, while these metabolites often have low expression or are absent in biotrophic pathogens. For example, the powdery mildew *Blumeria graminis* contains hardly any genes encoding biosynthetic enzymes for secondary metabolites (16), while in the biotrophic tomato leaf pathogen *C. fulvum* these genes are downregulated during infection (17).

Fungi protect themselves against antifungal proteins and metabolites that are present in plant cell walls and the apoplast by producing detoxifying enzymes such as proteases produced by *Fusarium oxysporum* f.sp. *lycopersici* (Fol) to degrade plant chitinases (18) or tomatinase produced by *C. fulvum* to detoxify the saponin α-tomatine to tomatidine (19).

PLANT DEFENSE

Plants have developed sophisticated defense strategies to recognize pathogens and to defend themselves against fungal pathogens. Recognition of chitin as a MAMP by PRRs induces MTI, an array of structural and chemical defense responses including callose deposition, accumulation of ROS, cell wall enforcements, and accumulation of PR proteins including chitinases, proteases, and glucanases that protect plants against potential microbial pathogens (20–22). PRRs are extracellular LRR-containing transmembrane proteins with a cytoplasmic kinase signaling domain known as RLK (23), S-RLKs, or extracellular membrane-localized LRRs that require association with RLKs to become activated by MAMPs. Successful pathogens have found ways to overcome these responses. They can suppress MTI by secreting different types of effectors that target various components of MTI (24–26). By suppressing MTI they achieve effector-triggered susceptibility. Alternatively, necrotrophic fungi produce effectors that induce necrosis and thereby create the dead tissue that supplies the nutrients the pathogens need. This is called NETS (9).

Effectors have mainly been described for various biotrophic but more recently also for hemi-biotrophic and necrotrophic fungi (6, 27, 28). All effectors manipulate host defense responses and host physiology to facilitate host colonization in various ways and are considered genuine virulence factors (6).

EFFECTORS PRODUCED BY OBLIGATE BIOTROPHIC, BIOTROPHIC, HEMIBIOTROPHIC, AND NECROTROPHIC FUNGAL PATHOGENS

Soon after Flor (29) postulated the gene-for-gene hypothesis, the search for the postulated gene products started. The hypothesis proposed that products of fungal avirulence genes interact with products of matching plant resistance genes, triggering defense responses often associated with an HR. The capacity to induce an HR facilitated the identification and characterization of Avr factors and cloning of their encoding genes. The *Avr9* gene of *C. fulvum* was the first fungal *Avr* gene to be cloned (30). Not much later the *Avr9* gene facilitated cloning of the matching *Cf-9* resistance gene, by a transposon tagging approach (31). Subsequently, multiple *Avr* genes complying with the gene-for-gene system have been cloned from different biotrophic and hemibiotrophic pathogens (6). Table 1 gives an overview of fungal effectors for which the function is known or for which a host target of a matching resistance gene has been cloned. Only those effectors for which a biological function has been found will be discussed in any detail.

Table 1 shows clearly that effector genes are very diverse and species specific. All effectors are secreted into the apoplast between host cells or into the extrahaustorial matrix (Fig. 2). Proteinaceous effectors of fungal pathogens are all secreted into the apoplast, where most of them function as inhibitors of basal plant defense. Many of them are SSCPs with even numbers of cysteine residues. In mature proteins they form disulfide bridges, making them resistant against multiple antimicrobial plant enzymes such as proteases, chitinases, or glucanases. Most effectors are also recognized by host immune receptors causing ETI. Thus, knocking out or silencing effector genes in different plant pathogenic fungi compromises not only avirulence on resistant cultivars with matching *R* genes, but also virulence on plants lacking these *R* genes (25). The expression of nearly all effector genes is strongly upregulated *in planta*, stressing their importance in virulence, likely due

Table 1 Effectors from obligate biotrophic, hemibiotrophic, and necrotrophic fungal pathogens whose intrinsic function, host target, or matching resistance gene is known[a]

Pathogen species and host	Lifestyle classification	Effector	Molecule	Molecular function/ structure	Localization in plant	Role in virulence	Resistance gene and protein (type)	Target	Reference(s)
Melampsora lini/flax	Obligate biotroph	AvrL567 (A, B and C)	Protein		Cytoplasm		*L5*, *L6*, and *L7* (TIR-NBS-LRR)		118
		AvrM	Protein		Cytoplasm		*M* (TIR-NBS-LRR)		117
		AvrP123	Protein	Kazal Ser protease inhibitor	Cytoplasm		*P*, *P1*, *P2*, and/or *P3* (TIR-NBS-LRR)		117
		AvrP4	Protein	Cystine knotted peptide	Cytoplasm		*P4* (TIR-NBS-LRR)		117
Blumeria graminis f.sp. *hordei*/ *barley*	Obligate biotroph	BEC4	Protein	Interaction with ADP ribosylation factor-GTPase-activating protein ARF-GAP proteins	Haustorium	Interference with host vesicle trafficking		ERF19	118
		Avra10	Protein		Probably in cytoplasm		*Mla10* (CC-NBS-LRR)		119
		Avrk1	Protein		Probably in cytoplasm		*Mlk1* (CC-NBS-LRR)		119
		CSEP0055	Protein	Binding to PR1 and PR17c	Apoplast	Required for penetration by secondary hyphae		PR1 and PR17	120
Cladosporium fulvum/tomato	Biotroph	Avr2	Protein	Cysteine protease inhibitor	Apoplast	Cysteine protease inhibition	*Cf-2* (RLP)	Rcr3 and Pip1	38, 121, 122
		Avr4	Protein	Chitin binding	Apoplast	Protects against chitinases	*Cf-4* (RLP)	Chitin	44, 45, 123
		Avr9	Protein	Cystine knotted protein	Apoplast	Unknown	*Cf-9* (RLP)		32, 124, 125
		Ecp6	Protein	Chitin binding (3 LysM domains)	Apoplast	Knock-down leads to reduced virulence		Chitin sequestering and perturbation of chitin receptor	126, 127
		Cftom1	Protein	Glycosidase	Apoplast	Deglycosylates α-tomatine into tomatidine; required for virulence		α-Tomatine	19

Ustilago maydis/maize	Biotroph	Cmu1	Protein	Inhibition of chorismate mutase required for SA biosynthesis	Cytoplasm	Inhibition of biosynthesis and plant defense responses		Chorismate mutase Cmu1	128
		Pep1	Protein	Inhibition of peroxidase-mediated ROS production by POX12	Apoplast	Required for pathogenic spread between host cells		Peroxidase POX12	129, 130
		Pit2	Protein	Cysteine protease inhibition	Apoplast	Reduced tumorigenesis		CP2, CP1A/B, XCP2 proteases	131
		Tin2	Protein	Enhances anthocyanin biosynthesis	Cytoplasm	Reduced supply of coumaric acid affects lignin biosynthesis		Protein kinase ZmTTK1	
Magnaporthe oryzae/rice	Hemibiotroph	Avr-Pita	Protein	Homology to metalloprotease, (not active enzyme)	Cytoplasm	Not required for virulence on rice	*Pi-ta* (CC-NBS-LRR)		49
		Avr-Pita2	Protein	Homology to metalloprotease, not active enzyme)	Probably in apoplast	Probably not required for virulence on rice	*Pi-ta* (CC-NBS-LRR)		132
		Avr-Pita3	Protein	Homology to metalloprotease, (not active enzyme)	Probably in cytoplasm	Probably not required for virulence on rice			132
		Pwl1	Protein	Glycine-rich hydrophilic protein	Probably in apoplast				133
		Pwl2	Protein	Glycine-rich hydrophilic protein	Probably in apoplast				134
		Ace1	Polyketide-nonribosomal peptide	Hybrid polyketide synthase/ nonribosomal peptide synthetase	Not secreted		*Pi33*		135
		Slp1	Protein	Chitin binding	Apoplast	Suppression of MAMP responses	*CEBiP* (RLK)	Chitin	136
		AvrPiz-t	Protein	Inhibition of RING E3 ubiquitin ligase	Cytoplasm	Suppression of PTI	*Piz-t* (NBS-LRR)	APIP6	53
Rhynchosporium secalis/barley	Hemibiotroph	NIP1	Protein	Stimulation of plasma-membrane H+ ATPase	Apoplast	Nonspecific toxin/ induces necrosis	*Rrs1*	Plasma-membrane H+ ATPase	93, 94
Fusarium oxysporum f. sp. *lycopersici*/tomato	Vascular wilt	Avr1 (Six4)	Protein		Xylem	Suppression of I-2 and I-3-mediated resistance	*I-1* (Not cloned)		137

(Continued)

Table 1 Effectors from obligate biotrophic, hemibiotrophic, and necrotrophic fungal pathogens whose intrinsic function, host target, or matching resistance gene is known[a] *(Continued)*

Pathogen species and host	Lifestyle classification	Effector	Molecule	Molecular function/ structure	Localization in plant	Role in virulence	Resistance gene and protein (type)	Target	Reference(s)
		Avr2 (Six3)	Protein		Xylem, and translocated to cytoplasm	Required for virulence; interacts with Six5 (Avr2)	*I-2* (CC-NBS-LRR); requires Six5 for recognition		137
		Avr3 (Six1)	Protein		Xylem	Required for virulence	*I-3* (S-RLK)		57
		Six5(Avr2)	Protein		Xylem	Required for virulence; interacts with Six3 (Avr2)	*I-2* (CC-NBS-LRR); requires Avr2 for recognition		58
		Six6	Protein		Xylem	Required for virulence; suppression of I-2-mediated HR, but not resistance	*I-2*		59
		FoMep1	Protein	Metalloprotease	Apoplast	Degrades plant chitinases with chitin-binding domain; required for virulence	Works in synergy with FoSep1		
		FoSep1	Protein	Serine protease	Apoplast	Degrades plant chitinases with chitin-binding domain; required for virulence	Works in synergy with FoMep1		
Verticillium/ tomato	Vascular wilt	Ave1	Protein	Activates Ve1-mediated resistance	Xylem	Required for virulence	*Ve1*		63
Pyrenophora tritici-repentis/ wheat	Necrotroph	ToxB	Protein		Apoplast		*Tsc2*		138, 139

Parastagonospora nodorum/wheat	Necrotroph	ToxA (also in *P. tritici-repentis*	Protein	Photosynthesis inhibition	Apoplast/ chloroplast	Unknown	*Tsn1* (NB-LRR protein kinase)	ToxA binding protein/ PR-1-5	140, 141
		Tox1	Protein	Chitin binding	Apoplast	Induces PCD; protects against chitinases	*Snn1* (Wall-associated kinase)	Chitin-binding protein	
Tox3	Protein		Apoplast	Induction of necrosis; binding to PR1	*Snn3*	PR-1-1			
Botrytis cinerea and other *Botrytis* species/many hosts	Necrotroph	NEP1-like	Protein	Cytotoxicity	Cytoplasm	Binds to host plasma membranes			98, 142
Small RNAs	RNA	RNA inhibition	Cytoplasm	Silencing of defense response genes		Argonaute	99, 143		
Cochliobolus heterostrophus/ maize	Necrotroph	*T-toxin*	Linear polyketide	Mitochondrial dysfunction	Cytoplasm	Increase of virulence		URF13	144
Cochliobolus carbonum/maize	Necrotroph	*HC-toxin*	Cyclic tetra-peptide	Inhibition of histone deacetylases	Cytoplasm	Suppression of defense responses	*Hm1* (mediates detoxification of HC-toxin by reduction)	Hm1/2	145
Cochliobolus victoriae/oat	Necrotroph	*Victorin*	Cyclic chlorinated penta-peptide	Induction of defense responses	Cytoplasm	Induction of defense-like responses	*Vb/Lov1*	Thioredoxin TRXh5	73, 74

[a]Blank entries indicate a knowledge gap.

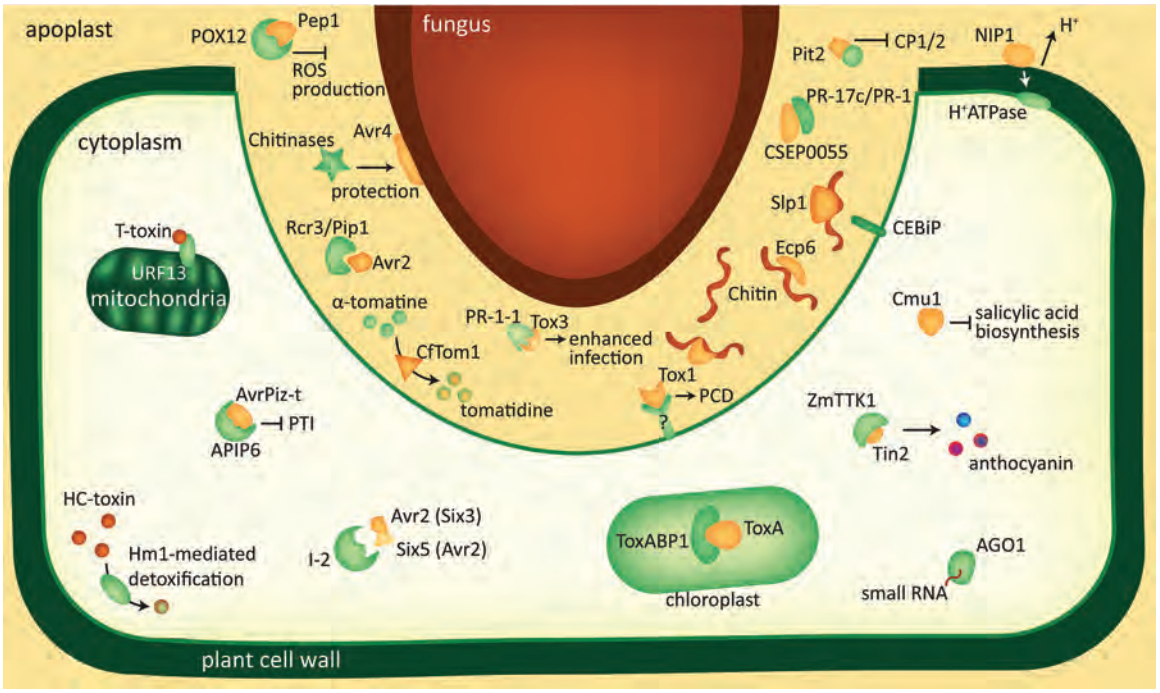


Figure 2 Interactions of fungal effectors with targets in plant host cells in biotrophic, hemibiotrophic, and necrotrophic diseases. The fungal cell interacts with the host cell via the yellow space, which represents either the extrahaustorial, matrix surrounding the haustorium, or the apoplast. Effectors from a range of fungi are presented in yellow, and their plant targets in green. Additional information is provided in Table 1. Adapted from reference 6.

to specific plant factors, but these have not been identified yet. For a few effector genes, nitrogen-limiting growth conditions induced their expression (32–34), but whether this reflects the *in planta* situation is uncertain.

EFFECTORS PRODUCED BY OBLIGATE BIOTROPHIC FUNGAL PATHOGENS

The gene-for-gene hypothesis was postulated by Flor (35) to explain the rust disease caused by the obligate biotrophic fungal pathogen *Melampsora lini* on flax. Several effectors from *M. lini* have been cloned, but their role in virulence is not known yet because transformation and targeted gene replacement have not been achieved for rust fungi. AvrP123 has structural homology with the Kazal Ser protease inhibitor, but whether this protein exhibits this function is not known. The same is true for AvrP4, which is a structural knotted peptide that is structurally related to the Avr9 protein of *C. fulvum*. Interestingly, the effector proteins from *M. lini* are translocated into the host cytoplasm, where they directly interact with TIR-NBS-LRR resistance proteins that are independent of the EDS1 defense pathway (36). Direct interaction between effectors and cytoplasmic immune receptors is the exception rather than the rule (37). Several effector genes have been cloned from the powdery mildew pathogen *B. graminis* f.sp. *hordei* including *Avra10* and *Avrk1*. These are recognized by cytoplasmic CC-NB-LRRs that trigger an EDS1-dependent defense pathway, but their intrinsic functions or host targets are not known. Two other *B. graminis* f.sp. *hordei* effectors have been described. BEC4 is secreted and reported to interact with ADP ribosylation factor-GTPase-activating proteins (ARF-GAP), while CSEP0055 binds to pathogenesis-related proteins PR1 and PR17c, whose functions are unknown. No plant immune receptors for BEC4 and CSEP0055 have been identified.

EFFECTORS SECRETED BY BIOTROPHIC AND HEMIBIOTROPHIC FUNGAL PATHOGENS

Effectors with Protease or Protease-Inhibiting Activities

The biotrophic extracellular tomato pathogen *C. fulvum* secretes several SSCPs in the apoplast of tomato leaves.

CfAvr2 inhibits the apoplastic tomato cysteine proteases Rcr3 and Pip1 (38, 39). Interaction of CfAvr2 with Rcr3 activates Cf-2-mediated defense in tomato (39). Interaction with the RLP Cf-2 is indirect and is a good example of the guard hypothesis, in which a resistance protein guards the host target of a fungal effector. Rcr3 is an important basal defense enzyme of tomato because it is also a target for secreted Oomycete (40) and nematode (41) effectors. Several effectors of other biotrophic fungi target plant cysteine proteases (42). Pit2 of *Ustilago maydis* inhibits the apoplastic maize cysteine proteases CP1 and CP2.

CfAvr9 has been suggested to be a carboxypeptidase-inhibiting protein because its structure is very similar, but carboxypeptidase activity has never been detected. CfAvr9 is recognized by the RLP Cf-9. Most of the SSCPs of *C. fulvum* have been reported to be recognized by RLPs (43). Interestingly, the biotrophic pathogen *U. maydis* secretes many effectors into the apoplast, where they exert their function or are translocated into the host cell, with or without a leader sequence, but none of them is recognized by resistance proteins in cultivated or wild corn species (6). During coevolution, they likely have become important core effectors after adapting or losing structural domains required for recognition by immune receptors.

Effectors with Chitin-Binding or Chitinase-Degrading Activities

Plant chitinases are crucial in basal plant defense as has become clear from the activity of several fungal apoplastic effectors. CfAvr4 contains a chitin-binding domain that protects fungal cell walls against hydrolysis by these enzymes (44, 45). Functional homologues of CfAvr4 occur in several related Dothideomycete fungi. *C. fulvum* Ecp6 binds chitin and does not protect against plant chitinases, but scavenges chitin fragments released by plant chitinases in the apoplast during infection and interferes with chitin-receptor heterodimerization, thereby suppressing chitin-induced basal defense responses. The wheat pathogen *Z. tritici* carries three Ecp6 homologues: Mg1LysM and Mg3LysM, which protect the fungus against plant chitinases, and Mg3LysM, which scavenges chitin fragments and suppresses chitin-induced basal defense responses (46). In addition, intercellular hyphae of *M. oryzae* secrete a chitin-binding protein, Slp1 (secreted LysM protein 1), which suppresses chitin-induced basal defense responses. The only cloned immune receptor that likely directly interacts with a chitin-binding protein is the tomato RLP Cf4, which matches CfAvr4 as well as other CfAvr4 homologues from other Dothideomycete pathogens (47). Interestingly, the necrotrophic fungal wheat pathogen *Parastagonospora nodorum* effector SnTox1 can also bind to chitin to protect the fungus against plant chitinases, indicating a dual function (48). *F. oxysporum* f.sp. *lycopersici* (Fol) secretes the metalloprotease FoMep1 and the serine protease FoSep1, which synergistically cleave tomato chitinases, reducing their antifungal activity and enhancing fungal virulence on tomato (18).

Apoplastic Effectors Inhibiting Other Apoplastic and Cytoplasmic Defense Enzymes

M. oryzae secretes the effectors Avr-Pita, Avr-Pita2, and Avr-Pita3, which all show homology to metalloproteases, but enzymatic activity has never been reported, nor has a role in virulence (49). They are recognized by the CC-NB-LRR immune receptor Pita (50). *U. maydis* secretes Pep1, which inhibits peroxidase POX12-mediated production of ROS. Fol secretes tomatinases that deglycosylate the saponin α-tomatine into the less antifungal β2-tomatidine (51), while *C. fulvum* secretes the α-tomatinase CfTom1, which deglycosylates and detoxifies α-tomatine into tomatidine (19). *U. maydis* secretes Cmu1, a chorismate mutase, to inhibit the production of the defense hormone salicylic acid (52). *M. oryzae* secretes AvrPiz-t, which is translocated into the plant cell and inhibits the RING E3 ubiquitin ligase APIP6 (53). Many more enzyme effectors are encoded by fungal genomes, but their role in virulence still needs to be determined (54).

The Effectors of the Vascular Wilt Pathogen *F. oxysporum* f.sp. *lycopersici*

A distinct group of pathogens colonize the xylem and induce wilt symptoms. The effector repertoire of Fol is well studied. Many Six (secreted into the xylem) proteins have been isolated from xylem fluids of Fol-infected tomato plants. Several of these Six proteins are Avr factors. Avr1 (Six4) is not required for virulence (in tomato) and is recognized by *I-1*, but it also suppresses *I-2*- and *I-3*-dependent resistance (55). Avr2 (Six3) is required for virulence and is translocated into the cytoplasm of host cells, where it is recognized by I-2 (56). Avr3 (Six1) is required for virulence and is recognized by I-3 (57). Also, Six5 is a virulence factor and is, like Avr2, recognized by I-2, but it requires interaction with Avr2 and vice versa for recognition by I-2 (58). In addition, Six6 contributes to virulence and suppresses I-2-mediated cell death but does not overcome I-2-mediated resistance (59).

Interestingly, Fol effectors are recognized by three types of immune receptors. Avr2 is recognized by I-2, a

cytoplasmic CC-NBS-LRR protein (60). Avr3 is secreted into the apoplast, where it is recognized by I-3, a cell surface-localized S-RLK (61), whereas the as yet unidentified Avr7 effector is recognized by I-7, a cell surface-localized RLP (62). I-7 provides resistance to races carrying Avr7, and its activity is not suppressed by Avr1. Avr1 is recognized by I-1 and encoded by a gene that has not been cloned yet, but it is also likely to be a cell surface-localized RLP. Interestingly, Avr1 inhibits ETI triggered by the Avr2/I-2 and Avr3/I-3 gene pairs, whose defense pathways are EDS1-independent, whereas Avr7-triggers an I-7-mediated defense pathway that is EDS1-dependent and cannot be inhibited by Avr1. It is also interesting that triggering of I-3-mediated resistance requires two effectors, Avr2 (Six3) and Six5 (Avr2), which interact with each other. The tomato vascular wilt pathogen *Verticillium dahliae* secretes Ave1 into xylem vessels, where it is recognized by Ve1, a cell surface RLP, (63). Ave1 shows homology with plant expansins and occurs in several other fungal pathogens and many plants (63, 64).

Effectors Secreted by Necrotrophic Fungal Plant Pathogens

Necrotrophic fungi are defined by their growth on dead or dying host tissues. Necrotrophs include species with broad host ranges, such as *Botrytis cinerea*, and these are predicted to produce effectors that are broad-spectrum phytotoxins. A second group of necrotrophs have narrow host ranges and produce a subset of effectors, now known as NEs, that contribute to virulence by inducing necrosis or chlorosis in specific genotypes of the single host species. Previously known as host-selective or host-specific toxins, these HSTs reproduce all or some of the disease symptoms induced by the pathogen itself (65). They are very diverse (Table 1) and include proteins and structurally diverse secondary metabolites. Most of these species are found in the Pleosporales family within the Dothideomycetes.

These NEs are involved in an inverse gene-for-gene interaction with host susceptibility genes, where the effector gene product interacts with the product of a matching host susceptibility gene, resulting in host susceptibility. Multiple studies now support that certain necrotrophic pathogens use the host's defenses to their advantage (66, 67) by tricking the plant into killing its own cells rather than the pathogen directly killing the plant cells. This can occur in a similar manner to *Avr* and *R* gene product interactions reported for several biotrophs, in that the NE and its recognizing host gene product interact, inducing an HR and local cell death. This prevents colonization of host tissue by biotrophic pathogens but provides a food source for necrotrophic pathogens. As such, the presence of both the NE and the matching host target leads to susceptibility, and absence of either the NE or the host target confers resistance. This strategy has been termed NETS (9).

Secondary Metabolite HSTs as NEs

The first HST to be discovered was AK-toxin in 1933, a necrosis-inducing epoxy-decatrienoic acid ester produced by the Japanese pear-infecting pathotype of *Alternaria alternata* (68). Deletion of one or more of the genes involved in the biosynthesis of AK-toxin resulted in loss of virulence (69). Since the discovery of AK-toxin, other pathotypes of *A. alternata* have also been found to produce secondary metabolite NEs. In each case the NE is the single determinant of pathogenicity and host range (70). The chemical structures of six of the seven *Alternaria* spp. HSTs have been determined, the exception being AT-toxin from the tobacco-infecting pathotype. AK-toxin and the toxins of the strawberry- and pear-infecting pathotypes, AT-toxin and ACT-toxin, are structurally analogous esters of a 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (70). The genes for the *A. alternata* HSTs are located on small chromosomes that are dispensable for growth, called conditionally dispensable chromosomes. Strong evidence has been obtained that these conditionally dispensable chromosomes can transfer horizontally between different *A. alternata* subspecies (71).

Victorin is the product of a nonribosomal peptide-synthetase produced by *Cochliobolus victoriae*, the fungal pathogen responsible for Victoria blight on oats (*Avena sativa*). Victorin induces cell death in certain varieties of oat, leading to susceptibility. The susceptibility gene, named *Vb*, has not been cloned, but strong evidence supports it being the same gene that confers resistance to crown rust, caused by the obligate biotroph *Puccinia coronata*. *Arabidopsis thaliana* also is sensitive to victorin (72), and the sensitivity gene *LOV1*, which encodes a NB-LRR repeat protein has been cloned (73). The inherent function of victorin is to inhibit a thioredoxin TRX-h5 that would normally provide a level of defense. Inhibition of TRX-h5 is not sufficient to promote disease. However, in the presence of LOV1, victorin induces defense responses that cause host cell death and lead to disease expression (74).

A related *Cochliobolus* species, *Cochliobolus carbonum*, causes northern corn leaf spot and ear rot on corn. *C. carbonum* produces a cyclic tetrapeptide called HC-toxin, which is responsible for increased virulence on susceptible corn plants (75). HC-toxin inhibits maize

histone deacetylases and thereby weakens the plant's defense. Susceptibility is conferred by lack of the *Hm1* gene, which encodes a reductase that detoxifies HC-toxin. *Hm1* was the first plant resistance gene to be cloned. While HC-toxin is produced by a necrotrophic pathogen and is considered an HST, it is different from the other effectors described here in that it suppresses rather than evokes host defense responses and in that susceptibility is conferred by the lack rather than the presence of a host gene.

Cochliobolus heterostrophus race T produces a polyketide toxin called T-toxin. Race T isolates are highly virulent on corn varieties carrying the Texas type male sterile cytoplasm (*T-cms*), due to an interaction between T-toxin and a mitochondrial membrane protein named URF13 present in these cultivars (76, 77). This interaction compromises the functioning of the mitochondria, resulting in cell death and increased virulence.

Proteinaceous Effectors Secreted by Necrotrophic Fungal Plant Pathogens

The NE ToxA, encoded by the gene *PtrToxA*, was identified in the wheat pathogen *Pyrenophora tritici-repentis* in the late 1990s and has since been arguably one of the most studied proteinaceous NEs (78). *SnToxA*, in the related pathogen *P. nodorum*, was identified due to its near perfect homology (99.7% amino acid identity) to the characterized *P. tritici-repentis* effector ToxA. Strong evidence supports this being an intriguing instance of recent (within the last century) horizontal gene transfer in which *P. tritici-repentis* was the recipient species (79). Different versions of SnToxA are found in different isolates of *P. nodorum*. The genes show signs of positive selection and possess varying levels of necrosis-inducing ability (80), indicating that modulation of the interaction with the host is crucial for isolate survival. All versions of SnToxA as well as PtrToxA interact with the same wheat recognizer gene *Tsn1*, a gene with a composite NBS-LRR structure (81). The direct or indirect interaction between of ToxA and Tsn1 plays a major role in susceptibility to both *P. nodorum* and *P. tritici-repentis*.

Sensitivity to ToxA results in necrosis and pathogen proliferation. In a susceptible plant, ToxA localizes to the mesophyll cells and binds to a chloroplast-localized protein named ToxABP1 and to plastocyanin (82). ROS then accumulate, followed by cell death. Determination of the ToxA crystal structure revealed a solvent-exposed loop harboring an arginine-glycine-aspartate motif (83), which is required for translocation into the host cell (84). Further details of the role of ToxA have come from metabolomics and proteomics studies which show that it induces a massive reprogramming of cellular metabolism and the induction of serotonin, an antifungal molecule that qualifies as a phytoalexin, a small molecule synthesized in response to a pathogen that has relevant antifungal activity (85, 86). The interaction between ToxA and Tsn1 is more recently reported to be mediated by an interaction with PR-1-5 (87).

The *P. nodorum* wheat disease has developed into a model system for understanding NE-based interactions. Three *P. nodorum* effector genes have, to date, been cloned. Following the identification of *SnToxA* in *P. nodorum*, a mapping and proteomics approach led to the identification of SnTox3—a 25.8-kDa protein with six cysteine residues that interacts with the recognizer protein *Snn3* (88). Subsequent studies showed that SnTox3 induces major biochemical defense responses including PR protein accumulation and the production of phenylpropanoid phytoalexins, but not serotonin (89). Like ToxA, Tox3 binds wheat PR-1 proteins. Tox3 specifically binds PR-1-1, leading to the formation of a peptide known as C-terminus of TaPR1-1 that enhances infection.

The identification of SnTox1 utilized the published genome sequence (90), using a bioinformatics scoring system based on the protein properties, genomic context, and interisolate comparisons to arrive at a list of putative effectors (91). SnTox1 is exceptionally cysteine-rich, because it contains 16 cysteine residues (13.7% cysteine). SnTox1 has a dual function in that it protects the fungus against host chitinases (48) while at the same time inducing defense-like responses in wheat carrying the *Snn1* susceptibility gene (92). The complexity of the interactions of ToxA, Tox1, and Tox3 with wheat belies the crude reputation of necrotrophic pathogens.

The NIP1 effector produced by the barley pathogen *Rhynchosporium secalis* is unusual because it is both an NE and an Avr factor (93–95). In the presence of the matching *Rrs1* gene, NIP1 is recognized and triggers defense responses and confers resistance. In the absence of *Rrs1*, NIP1 is a virulence factor, inducing necrosis (93–95). This is interesting, because both interactions induce necrosis but with different disease outcomes. Furthermore, different alleles of NIP1 induce differing levels of toxicity; however, the frequency of the different alleles in populations indicates that higher toxicity is not being selected for (93). This example hints at the complex nature of NEs, in that the timing and amount of necrosis may be important. Other NEs of *R. secalis*, namely NIP2 and NIP3, both induce necrosis but are not recognized as Avr factors.

NLPs AND SMALL RNAs SECRETED BY *BOTRYTIS* SPECIES

Botrytis species are pathogenic on both monocot and dicot plants. *B. cinerea* NLPs and homologous NLPs from other microorganisms are phytotoxic on many plant species. Two paralogous NLPs from *B. cinerea*, BcNEP1 and BcNEP2, produced in *Pichia pastoris* caused necrosis in many dicotyledonous plant species tested, but not in monocotyledons (95). Fluorescently labeled BcNEP1 and BcNEP2 proteins were associated with plasma membranes and the nuclear envelope, as well as in the nucleolus of responding plant cells. Strong accumulation of hydrogen peroxide was observed in chloroplasts. *Botrytis elliptica*, a pathogen of the monocot lily, also carries two *NLP* genes, *BeNLP1* and *BeNLP2*. Single knock-out mutants of *BeNLP1* and *BeNLP2* did not affect virulence of *B. elliptica* (96). Neither of the two BeNLPs produced in *P. pastoris* induced necrosis in lily. Because dicots but not monocots respond with necrosis after treatment with NLPs, these NLPs do not play a role in virulence on monocots. NLPs are under positive selection in *Botrytis* species, but their role in virulence on dicots remains to be elucidated (97).

sRNA effectors (98, 99) might play a role in the evolution of fungus-plant interactions. sRNAs are a new type of fungal effector that are delivered into host cells and suppress plant immunity by targeting *Argonaute 1* and thus disabling the host's RNA interference processes. Similar to effector genes, sRNAs are generated from repeat-rich regions of fungal genomes and appear to be subject to rapid evolutionary processes.

ORIGIN AND EVOLUTION OF EFFECTORS FROM FUNGAL PLANT PATHOGENS

Effector genes are subject to accelerated evolutionary processes we have termed "genomic tillage" (100). These evolutionary processes are selected because agri-ecological niches are subject to major and rapid (year-to-year) changes. New cultivars are regularly introduced and utilized in large monocultures to replace those whose resistance genes have been defeated. Furthermore, new agronomic techniques such as limited (or minimum or zero) tillage have had a major impact on crop diseases, and new fungicide classes are introduced about once every decade. Only those pathogens that can keep up with this rate of change remain able to cause significant diseases. The accelerated evolutionary processes include horizontal gene transfer, transposon activity, and RIP mutation, and they add to more familiar processes leading to point mutations and deletions.

It is no surprise, therefore, that many effector genes are located in repeat-rich regions that may have arisen through transposon activity and RIP mutation. This is most obviously illustrated by *Leptosphaeria maculans*, in which effector evolution is consistent with periodic episodes of transposon-induced RIP activity resulting in a highly compartmentalized genome (101, 102). Proximity to AT-rich repeats can increase gene mobility within the genome or between species (103) and could facilitate effector duplications, as are seen in the *PtrToxB* duplications in races of *P. tritici-repentis* associated with increased virulence (104) or the horizontal gene transfer of *ToxA* discussed above. Close proximity to AT-rich sequences has also been noted for some NEs. For example, *P. nodorum*'s *Tox3* gene is close to a region of AT-rich RIP-affected sequence around 10 kb long. Similarly, the genes required for the production of the *C. heterostrophus* secondary metabolite NE T-toxin exist within 1.2 Mb of AT-rich sequence (105).

As with Avrs, NEs have been observed to be under positive selection (97, 106, 107). In the *P. nodorum* wheat interaction, different ToxA isoforms vary in effector activity (108). This finding may be due to RIP activity, and it supported the hypothesis that variation at ToxA was due to selective pressure favoring increased virulence (108). In a similar manner to *Avr* and *R* genes, positive selection at NE and host recognition gene loci is probably indicative of the arms race between host and pathogen.

HGT was once thought to be an extremely rare occurrence or limited to species that were closely related and hence capable of normal gene-exchange processes. In addition to the HGT of the *ToxA* effector gene from *P. nodorum* into *P. tritici-repentis*, strong evidence exists that the *A. alternata* HST genes have transferred between the various pathotypes. Experimental demonstration of entire chromosome transfer between *F. oxysporum* f. sp. *lycopersici* and *Fusarium solani* f. sp. *pisi* has removed all doubt that this is a significant process. HGT has been shown to be more prevalent within the pathogen-rich *Pezizomycotina* subphylum than in other subphyla (109). Indeed, it seems certain that HGT has played a key role in the evolution of many pathogens and the recent emergence of certain crop diseases (110).

CONCLUSIONS AND FUTURE PERSPECTIVES

The first fungal protein effectors were discovered just over 2 decades ago (30, 77), although they were then called avirulence genes and host-specific toxins (65). Since then many more effectors have been discovered

together with their targets and recognizer genes. Estimates of the total number of effectors in a given species range from 20 to 200 (111), but given the somewhat arbitrary definition of effectors, the total number may be even higher.

Characterized effectors now include small RNAs, as well as small-molecular-weight compounds and proteins, and the latter are often posttranslationally modified (proteolytic cleavage, *N*-glycosylation, phosphorylation, etc.). They operate in the apoplast, the extrahaustorial matrix, the haustorium, the plant cytoplasm, or different cell organelles including chloroplast, mitochondrion, and nucleus. So far, we know they target plant antifungal compounds and antifungal defense pathways and camouflage fungal MAMPS, but this list of activities and targets is far from complete.

The isolation of effectors has significantly helped the identification of recognizer genes (31) and has also led to direct benefits to agriculture in a small number of cases (28). Knowledge of effector structure has been exploited to facilitate their discovery in related pathogens and in the selection of resistant host germplasm by plant breeders, but understanding and exploiting the overall complexity of the pathosystems remains a challenge (112).

Direct methods to exploit this knowledge for crop protection have so far proved rather elusive. While the general public's unease about genetically modified crops has not helped, the lack of broad-spectrum targets remains a real challenge. Effector families that are present in a wide range of pathogens, such as the Avr4 and Ecp6 families, may represent functions that could be targeted by a chemical, interfering RNA, or transgene. However, such families are rare, but we can expect that common functions will be found in more pathogens as more are studied. It is a fact of experimental life that many of the effectors cloned so far interact with resistance genes that are no longer effective in the field. The presence of both virulent and avirulent pathogens, and resistant and susceptible hosts, were essential experimental preconditions for the analyses. The ability of pathogens to evolve to overcome new *R* genes is well documented and is comparable to their ability to evolve resistance to fungicides (113). This ability is in part attributed to the location of effector genes in rapidly evolving parts of the genome (147) and in part to an unexpected propensity to acquire genes by horizontal transfer (100).

The identification of *L. maculans* effectors has enabled the rational deployment of resistance genes and has had a major impact on crop losses (114). Indeed, we can now acknowledge that our past practices of deploying resistance genes were as reckless as our deployment of antimicrobials directed against plant and animal pathogens. Knowledge of the structure of both effector and recognizer genes, coupled with vastly improved methods for the detection, differentiation, and quantification of pathogens, could be used in the stewardship of new *R* genes (whether genetically modified, genome edited, or conventional) (115). We can therefore conclude that the discovery of new effectors, their targets, and recognizer genes will all make major contributions to food security as well as reveal the exquisite complexity of plant fungal interactions.

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Emerging Fungal Threats to Plants and Animals Challenge Agriculture and Ecosystem Resilience

38

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INTRODUCTION

While fungi can make positive contributions to ecosystems and agro-ecosystems, for example, in mycorrhizal associations, they can also have devastating impacts as pathogens of plants and animals. In undisturbed ecosystems, most such negative interactions will be limited through the coevolution of fungi with their hosts. In this article, we explore what happens when pathogenic fungi spread beyond their natural ecological range and become invasive on naïve hosts in new ecosystems. We will see that such invasive pathogens have been problematic to humans and their domesticated plant and animal species throughout history, and we will discuss some of the most pressing fungal threats of today.

Terms Used in This Article

Accessory chromosome: A chromosome additional to the basic karyotype complement, that is, the complete set of chromosomes, which is not necessarily present in all strains of a given species.

Adaptive potential: The capacity of an organism to adapt in response to changes in its environment.

Agro-ecosystem: The organisms coexisting on agriculturally managed land, along with the agricultural environment, as it affects those organisms.

Clonal spread: Movement of a group of genetically identical individuals.

Ecosystem services: Tangible and intangible benefits or consequences of ecosystem function.

Elite germplasm: Seeds (or equivalent) of an organism, specifically optimized to a particular environment, usually through commercial breeding.

Emerging pathogen: A pathogen responsible for a disease that is appearing in a new host or in a new place or increasing its occurrence in an existing host.

Endophyte: Used in this context to mean a fungus that lives within plant tissues for at least part of its life cycle without causing apparent disease.

Evolutionary potential: Capacity of an organism to acquire and retain new alleles, or to undergo shifts in allele frequency, in response to selection pressure.

Extinction/extirpation: Extinction is generally considered to be the death of the last individual of the species, while extirpation is the loss of a species in the chosen geographic area of study, though it still exists elsewhere.

Generalist pathogen: Generalists thrive in a wide variety of environmental conditions and can make use of a variety of different resources (for example, different host plants).

Heterothallic: Species which have sexes that reside in physically separate individuals.

Horizontal gene transfer (HGT): Acquisition of genetic material by one organism from a second, often nonrelated, organism. May be direct or via a vector such as a virus.

Hypervirulence: Unusually high virulence.

Hypovirulence: Decreased virulence.

Innate immunity: Nonspecific defense mechanisms against pathogen attack of animals or plants, usually most relevant during the initial phase of that attack.

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Invasive species: An organism outside its native range, spreading rapidly and causing ecological and/or economic damage.

Long-distance dispersal: Spread of an organism to areas well beyond its current range, for example in international air currents.

Mycorrhiza: A symbiotic association between a fungus and the roots of a vascular plant.

Pandemic: An outbreak of infectious disease that spreads exceptionally widely: through populations, across countries, continents, or even globally.

Pathogenicity: The ability of an organism to cause disease in a particular host.

Obligate pathogen: Pathogens that must infect a host to survive, in contrast to other pathogens that are capable of survival independent of their host.

Saprophyte: A microbe that lives on dead or decaying organic matter.

Two-speed genome: A genome in which certain parts, which may range from specific, fairly small regions such as pathogenicity islands or entire (often accessory) chromosomes, are seen to evolve at a faster rate than the core genetic material.

Vertical transmission: "Inheritance" between generations; may refer to transmission of a pathogen or of genetic material (contrast to HGT).

Virulence: The severity of symptoms caused by a pathogen on a susceptible host.

Zoonoses: Disease of humans originating in animals and transmitted either directly or via a vector.

THE HISTORICAL IMPORTANCE OF FUNGAL DISEASE

Devastating fungal diseases of plants and animals can be identified throughout history. The decimation of harvests and ensuing famines, together with various efforts to prevent such outbreaks, are frequently described in early records. For example, wheat stem rust, caused by the fungus *Puccinia graminis* (still a problematic and invasive pathogen today; see "Current Examples of Emerging Infectious Fungal Diseases"), has been much feared for centuries (1). In the late 4th century BCE, in ancient Greece, Aristotle noted that this disease was favored by warmth and moisture (2). Meanwhile, the Romans held a festival, Robigalia, offering sacrifices to placate the god Robigo and protect cereal crops from such diseases (1). Around this time—circa 400 BCE—Hippocrates coined the words "endemic" and "epidemic" to distinguish between "normal" diseases such as coughs and colds and "abnormal" diseases such as plagues, in his Hippocratic corpus *Of the Epidemics*. References to plagues and pestilences of plants and peoples can also be found in the Bible as being heaven-sent curses or punishments.

Ergotism and Witchcraft

Fungal diseases of crops have, on occasion, led to some surprising social and political outcomes. A fascinating example is that of the ergot fungus, *Claviceps purpurea*. This pathogen of rye and other outbreeding cereal crops has a unique life cycle. Its sexual spores germinate on the stigmatic surface of its hosts' flowers, and its hyphae mimic pollen tube growth by extending downward to infect the ovary (3). The fungus then appropriates nutrients from the host to form a fruiting body, or sclerotium, in place of the developing seed. While fungal infection causes relatively little loss in yield, the grain quality is reduced due to the production of the fungal toxins ergotamine, ergine, and lysergic acid in the fruiting body (3). Ingestion of foodstuffs made from contaminated grain causes gastrointestinal symptoms followed by fatigue, depression, and painful muscular contractions. In more severe cases, vasoconstriction gives rise to "formication" (the feeling that the patient is covered in ants) and burning sensations and gangrene of the extremities, and psychoses may also occur (4). This suite of symptoms has long been recognized, with ergotism being dubbed "St. Anthony's fire" in the 11th century (5), but their source eluded mankind for centuries. The risk posed by ergot toxicity has diminished considerably since the identification of its fungal cause, with screening for contaminated seeds now in place. Nevertheless, there have still been isolated outbreaks, for example, in France in 1951 and in Ethiopia as recently as 2001 (6, 7). The most notorious case of ergot poisoning is perhaps that which triggered the 17th century witch trials in Salem, Massachusetts.

In a 17th century society dominated by political upheaval, puritanical Christianity, and social bickering, a group of young girls (aged 9 to 17) developed gastrointestinal symptoms, tingling and crawling sensations in the fingers, muscular contractions, mania, depression, and psychosis (4, 8). Unable to reach a diagnosis, a doctor suggested that the girls were bewitched (9). The patients then laid accusations of witchcraft against, initially, women on the fringes of the society (4, 8, 9). Fear and hysteria swept through the community and did not loosen its grip until 20 "witches" had been executed. At this time, similar symptoms were seen in Finnmark, Norway. Here, however, it was the patients themselves who were accused of witchcraft. A witch

hunt with even more tragic consequences than in Salem saw 137 people sent to trial, of whom around two-thirds were executed (10). In both Salem and Finnmark, it is thought that the illness which sparked the cycle of accusations, trials, and executions was due to ergotism (8, 9). Seasonal factors favoring the development of the fungus *C. purpurea*, including a cold winter followed by a humid spring, have been noted (4). Although other forces no doubt played a role in the extent of the accusations, it seems likely that *C. purpurea* triggered these ugly episodes, illustrating that fungi can influence human history in many ways.

Changing Landscapes: Chestnut Blight and Dutch Elm Disease

Emerging pathogens are those where the occurrence of the disease that they cause is entirely novel, or greatly increases, within a particular ecosystem. This may be due to the introduction of their host, either by man, due to a changed and newly permissive climate, or naturally; pathogen acquisition of new virulence genes or other traits allowing pathogenicity on a new host; or the appearance of a new genotype of a pathogen through hybridization, horizontal gene transfer, or other evolutionary events (discussed below in "Drivers for the Emergence of New Fungal Disease"). In the 20th century, two forest diseases emerged which destroyed large numbers of trees in Europe and North America. These serve as good examples of the damage done by emerging fungal pathogens in the recent past.

Chestnut blight is a disease of *Castanea* species, including the American chestnut *Castanea dentata*, the European *Castanea sativa*, and the Asian *Castanea crenata*. The causative fungus, *Cryphonectria parasitica*, is native to East Asia (11). Imports of *C. crenata* seedlings from Japan to North America appear to have been responsible for spreading *C. parasitica* to the United States (12). Once in North America, the fungus attacked native *C. dentata* trees. Within 50 years of the initial introduction of *C. parasitica*, mature *C. dentata* chestnut trees had been obliterated from the landscape. While uninfected rootstocks remain able to form new sprouts, these quickly become blighted, and no fruits are produced (11). *C. dentata* trees were previously the dominant canopy tree throughout the Appalachian forests (11), where their seas of white blossom held cultural importance. The loss of *C. dentata* trees also had profound ecological consequences, affecting both forest-floor and nearby aquatic habitats: chestnut leaves decompose easily and are of high nutritional value to invertebrates, compared to the leaves of the oak trees which largely replaced them (13). Impacts on mammal and bird species varied, with anecdotal accounts indicating a sharp drop in squirrel numbers due to the loss of mast production (14), while woodpeckers benefited from the increase in deadwood and insects therein (15). Meanwhile, seven moth species that lived or fed exclusively on chestnut became extinct (16). Thus, one fungus, *C. parasitica,* changed the forest landscape of the United States.

The spread of chestnut blight worldwide has been tracked by population genetic studies. In North America, genotypic data indicate that the source of the pathogen was Honshu, Japan, confirming the historical evidence of *C. crenata* seedling imports from this area in 1904. The American *C. parasitica* populations show considerable genetic divergence from the Asian populations; however, this is probably due to a small founder population, leading to random fixation of particular alleles in the emergent population (17). Overall, the data support a single or small number of introductions, followed by a rapid geographical spread. By contrast, *C. parasitica* spread slowly through Europe and without as marked destruction of the host *C. sativa* trees (17), which are less susceptible than American *C. dentata*. Additionally, European strains of *C. parasitica* themselves became infected with a virus, CHV-1, which caused fungal hypovirulence (17). Variation in the European fungal strains of *C. parasitica* is due to repeated introductions from both Japan and China. Despite the differences in spread pattern, American and European populations of the chestnut blight fungus show no genetic differentiation, indicating that there has been import and export of infected hosts between the two continents (17). Given the level of devastation the chestnut blight fungus unleashed in America—over 3 billion chestnut trees lost to disease—the fact that intercontinental trade in chestnuts was ongoing during this pandemic is, in retrospect, of grave concern.

A second fatal forest pathogen, which caused Dutch elm disease, spread across multiple continents in the 20th century, changing landscapes where grand elms once dominated the skyline. In contrast to chestnut blight, multiple pandemics of Dutch elm disease occurred. The final pandemic, in the 1970s, was the most damaging. Like the loss of American chestnuts, the loss of hundreds of millions of elm trees caused profound ecological changes, including the exposure of neighboring trees to storm damage and death and the creation of open woodland habitat that supported altered bird and mammal assemblages (11). The first pandemic, in the 1920s, was caused by the fungus *Ophiostoma ulmi*. This was a relatively weak pathogen, and it declined in disease severity naturally by the 1940s (18). However, a

second, more aggressive pathogen, *Ophiostoma novo-ulmi*, appeared during the 1970s (11, 19). This species has limited ability to interbreed with *O. ulmi*, and hybrid forms show reduced fitness. Nevertheless, transient hybrids occurred, and these provided a "genetic bridge" across which genes flowed between the species. These genes included a particular mating type allele, *MAT-1*, which increased the capacity of *O. novo-ulmi* to undergo sexual recombination, increasing its evolutionary potential (20). As we will see in "Natural Factors Promoting the Emergence of New Invasive Fungal Diseases," a high evolutionary potential is often important in the emergence and establishment of new infectious diseases.

Two subspecies of *O. novo-ulmi* exist, and both have invaded Europe: *O. novo-ulmi* subsp. *novo-ulmi* from the East and *O. novo-ulmi* subsp. *americana* from the West. These now hybridize freely (20). However, *O. ulmi* cannot coexist with the more aggressive *O. novo-ulmi*, so this pathogen is now locally extinct in many areas (20). As with chestnut blight, trade and transport are implicated in the spread of Dutch elm disease, with *O. novo-ulmi* subsp. *americana* reaching Europe in a consignment of logs sent from the United States to the United Kingdom in 1974 (20). In "Anthropogenic Factors Promoting the Emergence of New Invasive Fungal Diseases" we will discuss the importance of trade in plants and plant derivatives in the spread of agricultural and forest pathogens.

CURRENT EXAMPLES OF EMERGING INFECTIOUS FUNGAL DISEASES

Fungal diseases of plants have changed our landscapes and devastated our crops over the centuries. Since the Green Revolution, agricultural practices have favored intensive farming and the large-scale growing of hectare upon hectare of genetically uniform crops. Such plants are guarded from disease by one or two disease resistance genes or by the widespread spraying of fungicides. This plenitude of identical hosts has provoked widespread outbreaks of many known fungal foes on our major calorie crops. In this section, we focus on these emerging infectious diseases of domesticated crop plants and wild animals.

Diseases of Crop Plants

Soybean rust: *Phakopsora pachyrhizi* (Basidiomycete) (21–25)

What it infects

Soybean (*Glycine max*) and 95 other known legume species.

Becoming global

Sometimes called Asian soybean rust, this disease spread to Africa in the 1990s and then to South America and finally North America by 2004. Three main factors underlie its invasiveness: first, its asexual spores, which are produced in prolific numbers, can spread in global air currents. This includes cyclones and hurricanes, which cause unpredictability in terms of long-distance spread into new niches. These spores are then "rained out" of the atmosphere and deposited on wet leaves—ideal conditions for infection. Second, although *P. pachyrhizi* is a true obligate pathogen, it is a generalist: it can infect many different legume hosts. As well as soybean, it infects forage legumes such as vetch and lupin, as well as widely distributed weeds such as kudzu (*Pueraria lobata*), which is itself invasive. Third, *P. pachyrhizi* can rapidly generate new asexual spores by passing through its life cycle in as few as 9 days. Together, these factors ensure that the fungus spreads widely and finds a host readily. If soybean plants are absent, or only present seasonally, the pathogen sporulates on alternative hosts, repeatedly reinfecting newly planted soybean crops.

Why it matters

P. pachyrhizi causes an up to 80% reduction of soybean yields. This can lead to huge economic losses and social consequences, which have particularly affected parts of South America. An outbreak in 2001 in Paraguay caused severe losses and led to an outbreak in Brazil, in which up to 60% yield losses were reported. In 2002, over half a million metric tons of soybean were lost in Brazil alone. These losses, combined with the use of extra fungicides, cost Brazilian farmers 2 billion dollars during 2001 to 2003. The disease spread to Argentina in 2002 and Bolivia in 2003. In the United States, economic studies indicate that soybean rust could cause serious yield losses and increase production costs. In resource-poor farming areas, the resultant loss of livelihood leads to human suffering.

Mitigation

P. pachyrhizi is currently susceptible to treatment with azole and strobulurin fungicides. These target the fungal plasma membrane and interfere with mitochondrial electron transport, respectively. Prudent use of such chemicals, with careful monitoring, will hopefully lessen the risk of emergence of new, fungicide-resistant *P. pachyrhizi* strains. Moreover, meteorological monitoring and predictive modeling of global air currents and rainfall will aim to determine which crops are vulnerable and inform control measures.

Wheat stem rust: *Puccinia graminis* (Basidiomycete) (1, 26–32)

What it infects

Primarily a disease of wheat (*Triticum aestivum*), *P. graminis* can infect oats (*Avena sativa*), rye (*Secale cereale*), and barley (*Hordeum vulgare*), as well as the wild grasses *Festuca* and *Lolium* spp. The wheat stem rust fungus undergoes sexual reproduction on an alternative host, barberry (*Berberis vulgaris*).

Becoming global

Evidence suggests that the telial stage jumped onto grasses during their range expansion in the tertiary period, and the fungus was then further spread by agriculture. Barberry was identified as the alternative host in the 1780s. The fungus forms five spore types as it cycles between wheat and barberry. First, the teliospores, which have overwintered on wheat straw debris, germinate to form basidiospores which infect barberry, forming spermatia on the upper leaf surface. This triggers aeciospore formation on the lower leaf surface. These aeciospores are lifted into the boundary layer of the atmosphere and can travel thousands of kilometers before being deposited on wheat leaves, where they then form uredospores. These rust-red asexual uredospores are generated in prolific numbers and herald the start of epidemic disease levels. As the weather conditions deteriorate in autumn and the wheat harvests are gathered, the uredospore population declines in favor of the overwintering teliospores. Despite this complex dual-host life cycle, *P. graminis* is able to skip its telial stage to spread clonally in the absence of *Berberis*. Epidemics of wheat stem rust occurred in all wheat growing regions of the world during the 20th century; 60% of the U.S. wheat crop was lost in 1916, and eight more epidemics followed between 1920 and 1960. The popularity of Barberry as an ornamental in gardens and parks was implicated in these outbreaks. Meanwhile, Europe suffered epidemics in the 1930s, China in the 1950s, and Australia in the 1970s.

Why it matters

In addition to historical epidemics causing major yield losses worldwide, a new strain of *P. graminis*, known as Ug99, was discovered in Uganda in 1999. This new strain had spread to many African wheat growing countries, as well as Iran and Yemen, by 2005, and by 2014 had also been detected in Russia. There are now 11 known races of Ug99, each of which has broken the resistance offered by a different R gene bred into wheat. Ug99 is aggressive, causing significant yield losses, particularly to wheat varieties without effective disease resistance, and has shown itself to have high adaptive potential. It is feared that Ug99 will eventually reach other major wheat growing areas in Europe, Asia, and the Americas, causing catastrophic losses in global wheat yield.

Mitigation

Twentieth century epidemics were overcome by a combination of breeding for disease resistance in wheat and, in the United States, by the eradication of barberry plants. The former has mostly relied on gene-for-gene resistance, with durable protection derived from pyramiding of R genes. Destruction of over 50 million *Berberis* plants followed a major public education campaign ("Barberry or bread"), public participation, and legislation to make eradication compulsory.

Charcoal rot: *Macrophomina phaseolina* (Ascomycete) (21, 33–37)

What it infects

Over 500 host plant species are known. Individual strains show some degree of host specificity, but this is often incomplete, allowing the fungus to spread to infect other hosts. Crops infected by the charcoal rot fungus include soybean, sunflower, jute, cotton, cowpea, maize, sorghum, chickpea, peanut, canola, strawberry, guava, cassava, and melon. The fungus can also infect wild prairie grasses, as well as being an opportunistic human pathogen.

Becoming global

M. phaseolina was reported as the causal agent of guava wilt in India in 1990. It was identified as charcoal rot of strawberries in France in 1993 and of canola in the United States in 1994. In 1997, *M. phaseolina* was found causing stem rot of cassava in West Africa. In the 1990s, charcoal rot of soybean appeared in the United States. Charcoal rot of canola was found in Argentina in 2006 and Western Australia in 2009. Population genetics suggest this fungus spreads clonally, with its ability to infect wild species being important for its invasiveness.

Why it matters

M. phaseolina is a difficult fungus to identify. As such, it may have existed in many areas before it was first reported. However, charcoal rot began, more recently, to have to a serious impact on soybean yield in the United States. From 1996 to 2004, yield losses were estimated at around three quarters of a million metric

tons per year. Thus, *M. phaseolina* was the second most economically important disease of soybean at this time. In Africa's Sahel region over the same period, yield losses averaged around 10%; 100% yield loss is possible. The disease is most problematic in arid and semi-arid regions; the fungus thrives where soil nutrient content is low and temperatures are high. *M. phaseolina* is expected to become more geographically widespread under climate change models, and some host plants may be threatened with extinction. Increased occurrence of *M. phaseolina* is of further concern because it can act as an opportunistic mammalian pathogen.

Mitigation
M. phaseolina is hard to control, because its microsclerotia (hardened masses of fungal mycelium) persist in soil for up to 15 years. Soil treatment to mitigate the spread of fungal disease is problematic, not least because *M. phaseolina* shows resistance to many current fungicides. Moreover, antifungal prophylaxis does not prevent *M. phaseolina* infection in immunocompromised human patients, and the fungus can only be removed by wide surgical excision. Further research into this pathogen is needed, because its population genetic structure, life cycle, and manner of spread need to be better defined.

Leaf spot of barley: *Ramularia collo-cygni*, (Ascomycete) (38–41)

What it infects
This fungus primarily causes leaf spot disease on barley but is emerging as a pathogen of oats and wheat. It infects wild grasses including *Triticum, Festuca, Lolium, Elymus, Agropyron*, and *Brachypodium* spp.

Becoming global
R. collo-cygni was first described as a pathogen in 1893 in Italy, but rigorous scientific study of the fungus began in the 1980s. It was found in the United Kingdom in the late 1990s and detected in Europe, South and North America, the Middle East, Russia, Iceland, and Africa in the 2000s. This rapid emergence begs the question of whether it truly emerged or, instead, if improved recognition and detection led to its apparently sudden worldwide appearance. The latter seems a plausible explanation, because plants infected with *R. collo-cygni* remain symptomless while the fungus migrates to the awns (bristles on the glumes) and ears. The subsequent infection of the grains transmits the fungus from generation to generation and fuels further dispersal as infected germplasm is traded across the world.

Why it matters
This disease is largely symptomless in the early stages of infection, with symptoms appearing around the time of flowering and during grain set. This leads to low yield and low grain quality from a crop that appears uninfected until 3 weeks before the harvest is gathered. Recently, infections proved problematic in South America; high incidence and disease severity in Argentina in 2011 led to 70% grain yield losses, while in 2012, the fungus spread to six other South American barley growing areas, where it has now become a limiting factor in barley production. In 2002, widespread reports of barley leaf spot disease due to *R. collo-cygni* infection were reported in the United Kingdom. Hitherto, the fungus had been kept at bay by the use of strobilurin fungicides, but at this time, a point mutation known from related fungi rendered the fungus resistant to the quinone outside inhibitor fungicides. While the South American *R. collo-cygni* strain(s) has not yet acquired this mutation, if—or when—it does, grain costs and yield losses will rise.

Mitigation
Strobilurin fungicides are still in use in South America, but a limited lifetime should be expected and fungicide mixtures encouraged, given that fungicide resistance has arisen in *R. collo-cygni* elsewhere in the world. Trials have been conducted on seed treatments but without success, because the fungus behaves as a true endophyte, infecting the endosperm and embryo; treatments which kill the fungus are therefore detrimental to the seed itself.

Diseases of Animals

Amphibian chytridiomycosis: *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* (Chytridiomycota) (42–51)

What they infect
B. dendrobatidis has a broad host range, infecting all three classes of amphibian (anurans, caudates, and *Gymnophiona*). The fungus has a worldwide distribution occurring in 56 of 82 (68%) countries and infecting 42% of species. *B. salamandrivorans* has a more restricted range both in terms of its hosts, being found only in caudates (newts and salamanders), and geographic locations. Infection by these two chytrid fungi causes a cutaneous disease known as chytridiomycosis (Fig. 1).



Figure 1 Mass mortalities of midwife toads, *Alytes obstetricans*, caused by *Batrachochytrium dendrobatidis* (photo: Matthew C. Fisher).

Becoming global

Following the discovery in 1998 that *B. dendrobatidis* was a key driver of declines in amphibian species in Australia and the Americas, attention has focused on determining where outbreaks of chytridiomycosis have occurred and whether there are any underlying spatiotemporal patterns that indicate the original sources of infection, as well as pathways of spread. Phylogenomic analyses have shown that the worldwide emergence of chytridiomycosis is most likely explained by the rapid transmission of a hypervirulent lineage, called the global panzootic lineage (*Bd*GPL). This occurred on a global scale: this lineage has now been found in all continents alongside endemic lineages. Indeed, all mass-mortality and extinction events thus far attributed to chytridiomycosis caused by *B. dendrobatidis* are associated with the presence of *Bd*GPL. *In vivo* laboratory assessments of the virulence of *Bd*GPL have shown that this is indeed a hypervirulent lineage—a feature that confers "superbug" status on *Bd*GPL. However, the questions surrounding the origin of *Bd*GPL and the historical timing of this lineage's emergence across different continents remain elusive. In contrast, *B. salamandrivorans* is known to have emerged much more recently, having been most likely transported from Southeast Asia to Europe via the pet trade in infected amphibians.

Why it matters

Amphibians have long been recognized as being the most endangered vertebrate class on the planet, and chytridiomycosis is now recognized as a proximal driver of many amphibian die-offs worldwide. Indeed, chytridiomycosis has been described as "the worst infectious disease ever recorded amongst vertebrates, in terms of the numbers of species impacted and its propensity to drive them to extinction" (47). Thus, chytridiomycosis can result in widespread population decline, even extinction, in a subset of species and has contributed to the threatened status of almost 400 species of amphibian.

Mitigation

Countering disease-driven amphibian declines should consist of a multifaceted approach adapted to the stages of pathogen emergence. These include tackling the prearrival stage, the advancing invasion front, epidemic outbreaks, and established disease. Current approaches include prevention through restricting global trade in infected amphibians because it is clear that international movement of traded species is key to the spread of chytrids into naïve, disease-free ecosystems. Other short-term solutions include *ex situ* breeding programs known as "amphibian arks," alongside the cryopreservation of reproductive material. However, long-term, *in situ*, sustainable solutions are required if the goal of amphibian conservation is to be attained. This implies neutralizing the disease threat in wild populations using, for instance, antifungal compounds.

Bat white nose syndrome (WNS): *Pseudogymnoascus destructans* (Ascomycete) (52–55)

What it infects

First recognized in North America, *P. destructans* is now known to infect and cause clinical disease in at least six North American bat species. The fungus has also been described as infecting other species of bats in Eurasia and China.

Becoming global

WNS is a disease caused through infection of hibernating bats by *P. destructans*. First described following a cave die-off of little brown bats (*Myotis lucifugus*) in Schoharie County, New York, in the winter of 2006, the infection has since spread rapidly across eastern North America (Fig. 2). It was recently detected in Washington State, some 1,700 km from the index site. The mycosis is hypothesized to have been vectored into North America from Europe, where the fungus widely infects European bat species but where it does not cause observable mortality. Genetic analyses have confirmed that the North American variant of *P. destructans* is a single clone carrying a single mating type. This contrasts with the European genotypes, which are highly diverse and carry either one of the two heterothallic mating types.



Figure 2 Mass mortalities of little brown bats, *Myotis lucifugus* (photo: Alan Hicks).

Why it matters

P. destructans causes mass mortalities in North American hibernacula because the fungus destroys cutaneous membranes, including the wings, of hibernating bats. By 2015, four notable North American bat species' distributions had been entirely encompassed within the range of spread of *P. destructans*. As a result, species extirpations are predicted to occur in the near future.

Mitigation

Our current understanding suggests that the temperature of the bat hibernacula plays a role in WNS disease severity. Cooler and drier hibernacula appear to serve as refugia from disease impacts for some populations, because they carry lower fungal loads and thereby increase chances of bat survival. Indeed, it has been proposed that manipulating hibernacula entrances to create cooler and drier sites or restricting access to reduce bats' use of the warmer and wetter portions of hibernacula has potential as a single intervention in the management of WNS. Other approaches proposed include antifungal or biological (probiotic) interventions that could be used to reduce inoculum loads of *P. destructans* in the short term, thus allowing the bats time to adapt and evolve defensive/protective immunological responses to this pathogen.

Human cryptococcosis: *Cryptococcus neoformans* and *Cryptococcus gattii* (Basidiomycete) (56–60)

What it infects

Cryptococcus is a genus within the Tremellales, an order of fungi within the Basidiomycetes called "jelly fungi," due to their gelatinous fruiting bodies. They are commonly found growing on rotting wood as saprophytes. There are over 30 known species of *Cryptococcus*, of which two, *C. neoformans* and *C. gattii*, cause the majority of opportunistic human infections. Both species are readily recovered from the environment, where they can be isolated from the bark of a wide variety of tree species alongside other organic matter, most notably bird feces.

Becoming global

Genetic analysis has shown that *C. neoformans* and *C. gattii* have evolved independently from each other over the last approximately 30 to 40 million years. This separation has resulted in subtle variations in their virulence patterns. *C. neoformans* is the predominant species causing infection in immunocompromised individuals with HIV/AIDS. *C. neoformans* thus emerged in concert with the AIDS pandemic. *C. gattii*, however, causes rarer infections in putatively immunocompetent individuals. An unusual cluster of cryptococcal disease

in otherwise healthy individuals was seen on Vancouver Island, British Columbia, in 1999. This infection, caused by *C. gattii*, has now expanded into mainland Canada and the northwestern United States and is known as the Pacific Northwest outbreak. It was likely seeded by infectious inocula from South America.

Why it matters

The virulence attributes of *Cryptococcus* stem from various adaptations it has undergone that allow it to survive in the environment. An array of recognized "dual-use" survival/virulence factors is known. The most notable of these is the thick enveloping polysaccharide capsule, which in the environment defends against attack by parasitic amoebae but which also allows the fungus to survive macrophage assault and to disseminate as an intracellular parasite within the human body. Such adaptations have led to widespread human infection where the prevalence of HIV is high. In 2009, the CDC estimated that there are close to one million cases per year, with at least 100,000, and perhaps 500,000, deaths per year in sub-Saharan Africa alone, where cryptococcal meningitis accounts for around 17% of AIDS mortality.

Mitigation

The most important method of controlling cryptococcal meningitis is to control HIV. In Europe and North America, the numbers of cryptococcal patient cases fell dramatically after introduction of effective antiretroviral therapy. However, despite the widespread use of antiretroviral therapy in sub-Saharan Africa, there is no evidence of a reduction of cryptococcal infections. This may be due to the emergence of antiretroviral therapy resistance in target populations provoked by nonadherence to treatment regimes. The recent introduction of a cheap and easily used antibody-based disease diagnostic "dipstick" test that detects cryptococcal antigen holds much promise. This test has the potential to allow earlier treatment of the disease with potent antifungal drugs. It has triggered the widespread advocacy calling for the affected populations to have access to the most advanced antifungal drugs at an affordable cost.

Azole-resistant aspergillosis: *Aspergillus fumigatus* (Ascomycete) (61–63)

What it infects

The genus *Aspergillus* comprises a few hundred fungal species found in various climates worldwide. Certain *Aspergillus* species cause a variety of clinical diseases in humans and animals. The disease spectrum ranges from allergic conditions such as allergic bronchopulmonary aspergillosis through to invasive tissue disease in immunosuppressed tissue-transplant patients. Allergic bronchopulmonary aspergillosis is primarily seen in patients with chronic *Aspergillus* colonization suffering from cystic fibrosis and chronic granulomatous disease. Patients with lung diseases such as chronic obstructive pulmonary disease can develop chronic pulmonary aspergillosis when the fungus grows and becomes locally invasive, thus causing tissue damage.

Becoming global

The triazole antifungals, including itraconazole, voriconazole, isavuconazole, and posaconazole, are widely used to treat diseases caused by *Aspergillus* infection. However, azole-resistant strains have recently emerged across six continents and pose a significant new therapeutic challenge. Although *de novo* azole resistance is known to have arisen occasionally in patients during azole therapy, the main burden is now known to have been caused by the acquisition of resistance from environmental isolates. Here, the evolution of azole resistance in *A. fumigatus* is attributable to the widespread use of azole-based fungicides, which are widely deployed to control fungal disease in agriculture. The resistance phenotype is associated with particular key mutations in the fungal *cyp51A* gene. The occurrence of rapid selective sweeps associated with such new alleles has been documented.

Why it matters

cyp51A-mediated resistance is increasing rapidly; in some settings up to 10% of patients with *Aspergillus*-related disease do not respond to azole treatment. The prognosis for these patients is poor, with up to 80% mortality.

Mitigation

Azole fungicides are widely used in agriculture; they rank as the most important antifungal from a global perspective. Worldwide measures to curb their use should now be considered due to their attendant selection for antifungal resistance on nontarget pathogenic fungi such as *A. fumigatus*. But intervention is problematic, because azole use is so widespread in agriculture. A ban on azole use in crop disease prevention would alter reliance on other classes of fungicide, such as strobilurins and fast-forward emergence of new fungicide-resistant genotypes. However, this highlights the need either to reserve the antifungal azoles for the treatment of human fungal disease or to develop new antifungal drugs for human use.

DRIVERS FOR THE EMERGENCE OF NEW FUNGAL DISEASE

Natural Factors Promoting the Emergence of New Invasive Fungal Diseases

Most organisms are able to resist infection by most potential pathogens through innate immunity (e.g., 64). Pathogens, in turn, constantly evolve to overcome host defenses, creating selection pressure for hosts to withstand such pathogens. There is thus a classic evolutionary arms race between host and pathogen (65). While it is relatively easy to understand this process, it is considerably harder to predict when, where, and how new diseases will emerge.

The key factor determining the likelihood that a microbe will become pathogenic for the first time, on a new host or in a new place, is its "evolutionary potential." This is the organism's potential for genetic adaptability, and it is underpinned by a combination of the organism's biology, genomics, and population genetics. First, variability is a prerequisite for selection. A linked factor is effective population size, because more reproducing individuals generate more mutations on which selection can act. Second, reproductive mode affects evolutionary potential, because recombination creates new mixtures of genes (66, 67). Third, certain features of pathogen genomes may add to their evolutionary potential, increasing recombination, hybridization, variability, or rates of horizontal gene transfer (67, 68) (Fig. 3).

The biology and life cycle of the wheat pathogen *Zymoseptoria tritici* (Septoria leaf blotch disease) exemplifies all of these factors (Fig. 4). This fungus shows plentiful genetic variation, with evidence of gene flow between populations (69). It causes a polycyclic disease, with many cycles of asexual reproduction occurring within a wheat growing season, which allows selection for virulence (70). However, *Z. tritici* also reproduces sexually (71). The fungal genome consists of a core set of chromosomes and a suite of up to eight accessory chromosomes of unknown function, carrying genes which are largely silenced (72). The complement of these accessory chromosomes varies with fungal strain and can also change between generations. Further, there is evidence of multiple insertion and deletion events occurring in the accessory chromosomes. The changes in accessory chromosome number probably originate through nondisjunction at meiosis, followed by the fusion of sister chromatids. This leads to chromosomal degeneration and is probably the origin of the accessory chromosomes. The rapid evolution that takes place on accessory chromosomes (73) contributes to the pathogen's adaptive potential (74) and helps deleterious changes to be lost while advantageous ones are preserved. Other hallmarks attesting to the plasticity of this fungus are seen in its genome, which contains 17% repetitive DNA, 70% of which is enriched in class 1 transposable elements, which are associated with high mutation rates (75). *Z. tritici* poses a major threat to wheat production throughout the temperate world (71, 76; see "Threats to Agriculture from Emerging Fungi"). Its ability to evolve rapidly not only challenges our ability to breed for durable disease-resistant wheat cultivars but also promotes the emergence of fungicide resistance.

Genomes like that of *Z. tritici*, where accessory chromosomes are associated with high evolutionary potential, are often termed "two-speed" genomes. This refers to the separation of their core set of slowly evolving, essential genes from their accessory genes, genomic regions, or chromosomes, which are enriched in repetitive DNA, transposable elements, or other features which make them hot spots for mutation, recombination, and even for the introgression of genes from other species (77). These rapidly evolving genomic regions are frequently associated with pathogenicity and may be lineage-specific, determining host range (74). Examples can be seen in the genus *Fusarium*, a collection of saprophytic and pathogenic fungi which can infect both plants and animals, including humans. *Fusarium solani*, also called *Nectria haematococca*, carries up to three accessory chromosomes, which include genes more similar to *Aspergillus* genes than to the rest of the *F. solani* genome (76). One such accessory chromosome encodes pathogenicity factors that allow the strain which carries it to infect pea plants, having degraded a host defense compound (a phytoalexin) (76, 78). The related generalist *Fusarium oxysporum* (Fig. 5; see section on Panama disease) also carries accessory chromosomes containing many transposable elements and virulence genes which define its host range. These accessory chromosomes can be readily exchanged, even between fungal strains which are not sexually compatible and between strains of different species in the genus *Fusarium* (79). Not all *Fusarium* species carry accessory chromosomes, but even those lacking this feature have rapidly evolving genomic regions associated with pathogenicity and virulence determinants (80). The wheat pathogen *F. graminearum*, which has recently re-emerged to cause worldwide problems (69), has four chromosomes that show high single nucleotide polymorphisms and rapid recombination rates in the telomeric regions at the ends of chromosomes, reflecting ancient chromosome fusions (81).

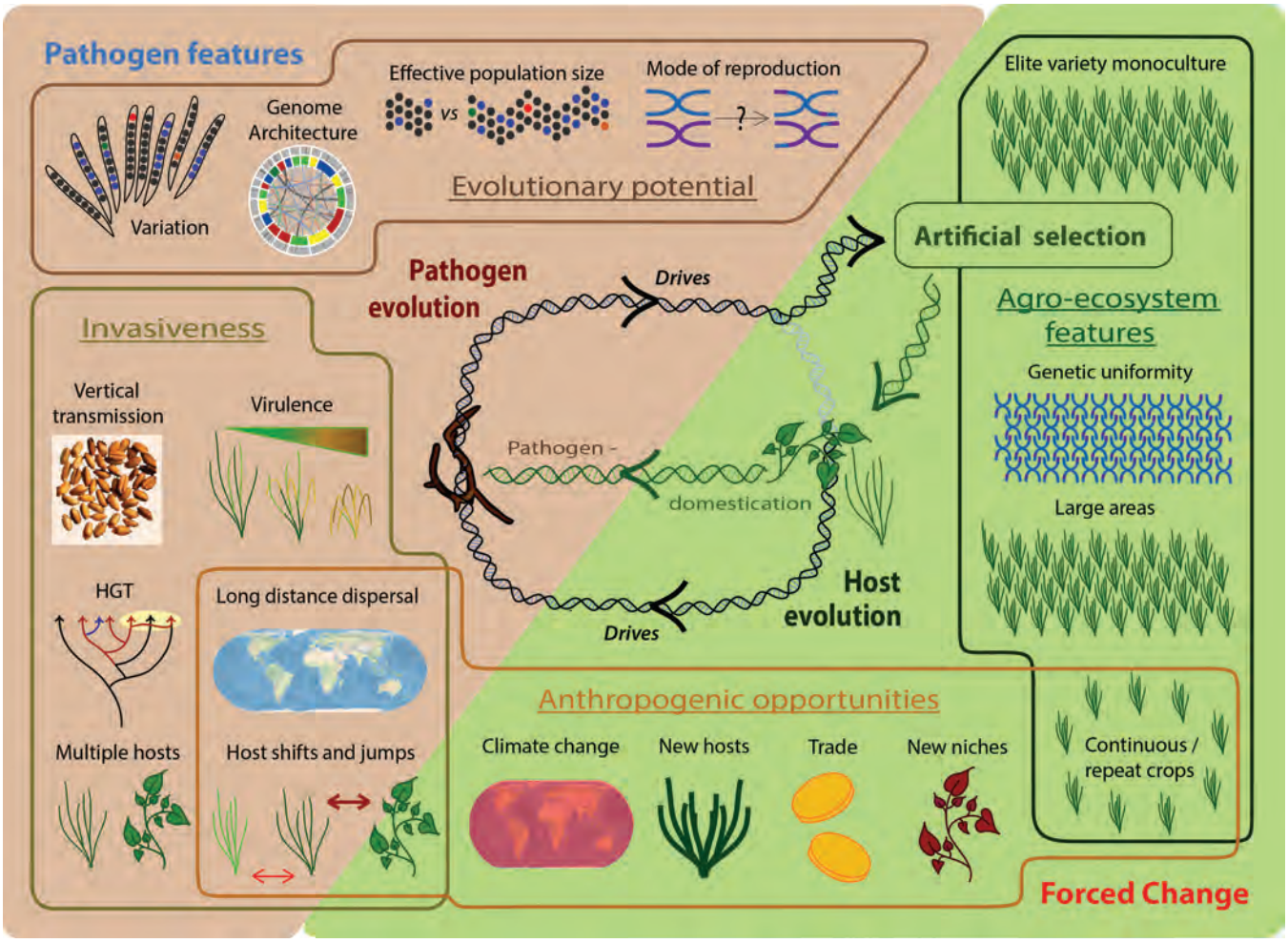


Figure 3 Factors influencing the emergence of infectious diseases of crop plants. These include features of the pathogens themselves as well as changes forced by the opportunities given to and pressures placed upon pathogens externally, as a result of human activity. Pathogen features prompting the emergence of new diseases include specialized genomes, sexual reproduction, large populations, and plentiful variation, which contribute to evolutionary potential. Invasiveness is key to the appearance of new emerging infectious diseases. This pathogen trait has natural components including high virulence and the capacity to infect multiple hosts and to transmit vertically, as well as such traits as long-distance dispersal and a propensity to undergo host shifts and jumps. Such traits may be natural or appear as a result of opportunities provided by anthropogenic changes to the environment. Such anthropogenic opportunities include climate change and trade and transport, introducing pathogens to new places, niches, and hosts. They may also take the form of pressures placed on pathogens by the continuous cultivation of a single crop year-round leading to genetic uniformity of available hosts over large areas. Such elite varieties are the product of artificial selection, which is the main driver of host evolution when the host is a domesticated crop. Host evolution can then drive pathogen evolution, leading to the appearance of new emerging infectious diseases and subsequent selection of new, resistant elite varieties. In addition, during the domestication of a crop, pathogens may be directly domesticated, short-cutting their adaptation to a particular host.

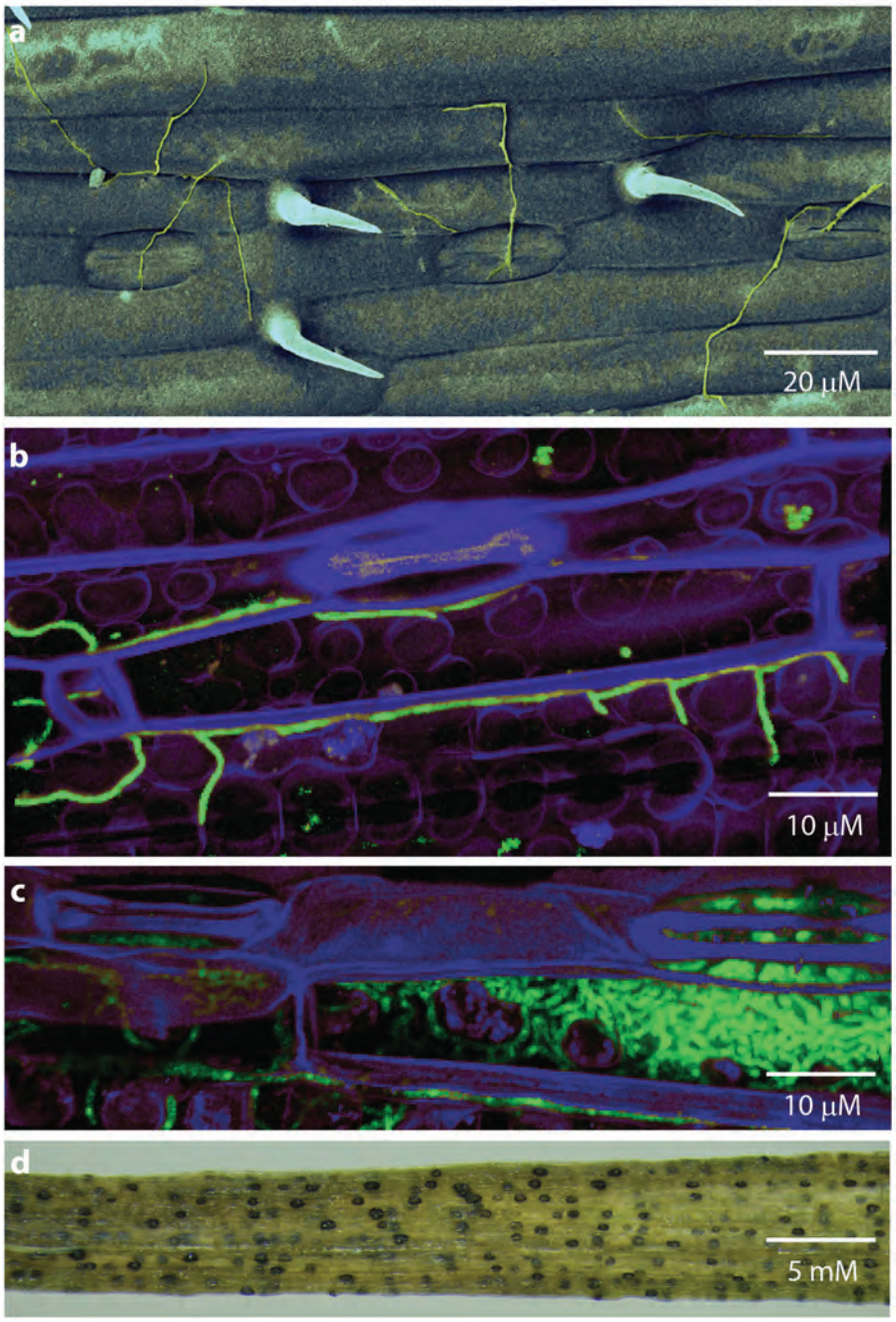


Figure 4 The wheat pathogen, *Zymoseptoria tritici*. (**a**) Scanning electron micrograph showing *Z. tritici* (green) on the surface of a wheat leaf. False color image. (**b**) Confocal fluorescence micrograph showing green-fluorescent protein-tagged *Z. tritici* (turquoise) beginning to colonize mesophyll tissues of a wheat leaf (purple). False-color image. (**c**) Confocal fluorescence micrograph showing *Z. tritici* (turquoise) proliferating with a wheat leaf at a late stage of infection. The fungal mass on the right of the image is a nascent pycnidium, the structure in which sporulation takes place. (**d**) Severely diseased wheat leaf showing *Z. tritici* symptoms, including mature pycnidia (black). Each black spot can release hundreds of spores. (Photos: Helen Fones; GFP-tagged *Z. tritici*: reference 127.)

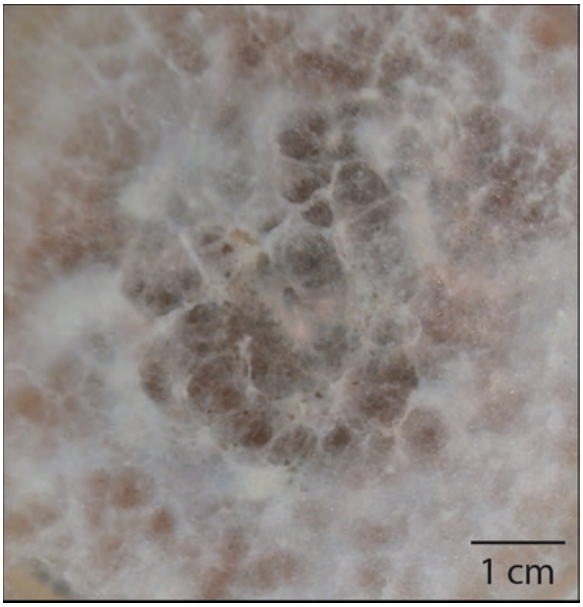


Figure 5 *Fusarium oxysporum* f. sp. *cubense* race 4 (Panama disease). Culture on potato-dextrose agar in a laboratory, showing characteristic purple coloring. (Photo: Helen Fones.)

The exchange of accessory chromosomes between *Fusarium* strains indicates that they undergo some form of HGT. HGT is common among bacteria and frequently allows the transfer of pathogenicity, virulence, or antibiotic resistance genes, creating "pathogenicity islands" in bacterial genomes. HGT had been assumed to be rare in eukaryotes, but new evidence concerning exchange of pathogenicity genes in particular suggests that it may be much more commonplace in filamentous fungi than realized. Such evidence usually comes from comparative genome data, the availability of which has recently exploded with the advent of rapid DNA sequencing technologies and analytical tools. Nucleotide sequences gained through HGT, including bacterial pathogenicity islands, can usually be recognized by a GC content different from the surrounding region of the genome and by a nearby abundance of transposase genes (encoding enzymes that move transposons). The *F. solani* chromosome containing the pea pathogenicity gene conforms to this pattern (76). There is a similar HGT footprint in the *toxA* gene of the notable wheat pathogen *Pyrenophora tritici-repentis*, which appears to have transferred from *Phaesosphaeria nodorum*, and in a cluster of pathogenicity-related genes in the maize smut pathogen *Ustilago maydis*. In the generalist plant pathogenic fungus *Alternaria alternata*, which provokes human allergies, such clusters are known to define host range (78).

So what are the endogenous attributes which favor the emergence of a new pathogen? Pathogens with large populations, high mutation rates, high rates of outcrossing and genetic recombination, and a high level of genomic plasticity, including the ability to exchange genetic material in a nonsexual manner such as HGT, are most likely to emerge as new pathogens: they have the evolutionary potential to overcome host defenses in a naïve plant or animal (79). Various routes have been identified for this emergence. Chief among these are host jumps and host shifts (82). It is relatively easy to see that the acquisition or loss of an accessory chromosome or a single or set of pathogenicity genes could lead to a host jump, by allowing a pathogen of one host suddenly to infect another. Historical examples of host jumps include the rice blast pathogen, *Magnaporthe oryzae*, which jumped from Setaria millet onto rice 7,000 years ago (78), and the ergot fungus, *C. purpurea* (see "Ergotism and Witchcraft"), whose closest relatives are symbionts of wild grasses and pathogens of arthropods (83, 84). More recently, the rice blast fungus jumped onto wheat in Brazil and, in 2016, onto wheat in Bangladesh (85, 86). Host shifts are smaller, between closely related hosts. Another route for the emergence of a new pathogen is through hybridization between two or more related fungal strains. An example of this mechanism was seen in Dutch elm disease (see "Changing Landscapes: Chestnut Blight and Dutch Elm Disease"), where hybridization between *O. ulmi* and the new, more aggressive *O. novo-ulmi* allowed the transfer of pathogenicity genes from the older species into the emergent, more devastating pathogen (20, 78). Other hybrid emerging fungal pathogens include poplar rust, *Melampsora columbiana*, and *Botrytis allii,* a postharvest disease of onions (20).

In addition to these factors, which promote the emergence of a new pathogen, there are also natural factors which play a role in determining pathogen invasiveness. Earlier in the article we saw that certain invasive plant pathogenic fungi share the ability to travel internationally in air currents (22, 23, 30). This allows worldwide spread of spores to infect any susceptible host population. In addition, it allows obligate pathogens to repeatedly reinvade areas where the host is not available year-round (87). Airflows are largely predictable, in both a local and global sense, allowing models to identify major risk areas based on pathogen source populations, spore release times, and prevailing air currents. But there are also unpredictable, extreme weather events such as cyclones or hurricanes (12, 87, 88) which can carry pathogens to new areas faster than expected. Interestingly, it now appears that for

foliar crop pathogens, this long-distance dispersal is not a special adaptation, but rather an extension of the processes involved in short-distance spread among hosts within a field, combined with turbulent airflow at small spatial scales (87). Fungal foliar crop pathogens appear to be uniquely successful at long-distance dispersal, which may be an exaptation derived from their usual modes of transmission (primarily rain splash and wind dispersal) (87). Similar processes are, however, also probably contributing to the aerosolization and contemporary dispersal of azole-resistant *A. fumigatus*, leading to the occurrence of the resistant *cyp51A* alleles in regions where there is little drug pressure (61).

An alternative—or additional—method of spread involves passage from one host generation to the next. For crop pathogens, this means transmission via the seed. This is particularly effective when both infected seeds and young plants appear healthy. Infected seeds allow the pathogen to use the host's own dispersal mechanisms while ensuring a susceptible host on arrival. International seed trade amplifies the effectiveness of this strategy (see "Anthropogenic Factors Promoting the Emergence of New Invasive Fungal Diseases"). An example of this vertical transmission is *R. collo-cygni*, which as we saw above, lives endophytically, causing no symptoms, until the plant begins to flower (40). There is a trade-off in virulence between intra- and interhost replication (78); endophytic growth is a solution which precludes any risk of host death before transmission.

Another feature of fungal pathogens which promotes their invasiveness is the ability to establish a year-round infection in a new area, often taking advantage of volunteer or wild plants. Such continuous infection means that there is a continuous inoculum source in the new location, even if the crop is not grown year-round. Alternatively, establishment of a year-round infection on crop plants in an area where they are grown year-round can provide year-round inoculum for invasion of nearby areas where the same crop is seasonal. Again, this ability is shared by many of the fungi discussed in "Current Examples of Emerging Infectious Fungal Diseases." There are a number of ways fungi achieve this. Broad host range, as seen in *M. phaseolina* and *Fusarium* spp., is one strategy; generalist pathogens dispersing to a new area have a range of potential hosts available and are thus likely to be able to become established. The same effect can be achieved, however, by more specialist fungi with narrower ranges of alternative hosts if those hosts are themselves widespread. The fungi *R. collo-cygni* and *P. graminis* exemplify this strategy, because they infect a number of wild grass genera whose species are found throughout temperate regions (1, 40, 41). Similarly, *P. pachyrhizi*, while primarily studied as a soybean pathogen, is able to infect many legumes, including the invasive weed kudzu, whose large, widespread populations greatly increase host availability (23, 24). Alternatively, fungi may form resting spores (such as the microsclerotia formed by *M. phaseolina*), which can survive cold winters or persist through hot or dry summer periods. Such a strategy increases the chances of infecting any hosts that become available in a new environment as the seasons change.

Anthropogenic Factors Promoting the Emergence of New Invasive Fungal Diseases

In addition to the factors outlined above, some human activities affect the likelihood of new invasive fungal diseases emerging. The most important of these is trade and transport (11), followed closely by agricultural practices that lead to wide distribution and high-density cultivation of genetically uniform plants or livestock, susceptible to the same diseases (66).

In "Changing Landscapes: Chestnut Blight and Dutch Elm Disease," we saw that the international trade in chestnut and elm continued during both the chestnut blight and Dutch elm disease epidemics. In agriculture and sylviculture, trade in products such as food or wood is commonplace, as is the worldwide trade in germplasm—including seeds, rhizomes, or cuttings of elite crop varieties. In "Natural Factors Promoting the Emergence of New Invasive Fungal Diseases," we saw that seed-borne fungi can exploit both natural and anthropogenic seed dispersal mechanisms. Live plants and animals are also traded and may carry a range of pests and pathogens undetected into new areas. Such trade is common in asexually propagated crops such as banana (see section on Panama disease). Trade in live plants is implicated in the spread of the ash die-back fungus (*Hymenoscyphus fraxineus*; see "Threats to Natural Ecosystems from Emerging Fungi") to the United Kingdom (89) and white pine blister rust fungus (*Cronartium ribicola*) to the United States, where the host, *Pinus albicaulis*, is now threatened, and Canada, where it is endangered (90, 91). Similarly, it is clear now that the international trade in Asian salamander species is enormous, with, for instance, over 2.3 million individuals of *Cynops orientalis* being imported into the United States from 2001 to 2009 (43). There has been much focus on how the transcontinental vectoring of *Bsal* in amphibians may have occurred. Molecular surveillance data have demonstrated

that *Bsal* is relatively widespread in European captive collections (45) and is associated with Asian species as well as with outbreaks of disease in captive European species of caudates. A clear case has therefore been made that the international movement of traded species of amphibians is a key mechanism that is contributing to the spread of *Bsal* and its invasion of naïve, disease-free ecosystems. These potent arguments have recently resulted in a ban on the importation of 201 salamander species into the United States (92).

When potential hosts are introduced to a new area, there are a number of associated risks. First, the imported organisms may be infected with a disease not occurring in the new location. Such introductions of deleterious organisms are sometimes termed "pathogen pollution" (11). Second, the introduced organism can carry a pathogen or commensal organism that has previously caused little or no disease but which may, in the new location, acquire new virulence or pathogenicity genes via HGT or hybridization, creating a new pathogen. Third, such fungi might possess or obtain genes for virulence in hosts endemic to the new area. These risks are compounded by the fact that closely related species of fungi which have diverged allopatrically (while occupying separate, nonoverlapping geographic areas) will not have experienced selection pressure toward perfect reproductive isolation. Thus, reproductive barriers may be leaky, allowing the formation of "hopeful monsters" (93)—hybrids which, while usually having lower fitness than the parents, occasionally bring together pathogenicity and virulence determinants in devastating new combinations. Backcrossing to either parent can then fix these genes in otherwise well-adapted local pathogen populations. Fourth, climatic factors might favor an imported pathogen or potential pathogen such that it is no longer limited by hot summers, cold winters, or drought. Finally, biotic factors may favor an introduced organism, because by invading new territories it may leave behind competitors, predators, or parasites, thus undergoing "enemy release." This allows the invader population to explode in numbers, further increasing the likelihood of favorable HGT, hybridization, recombination, or mutation events. Anthropogenic transport and mixing of pathogens can thus have serious consequences (11, 69, 87, 89–94).

Another anthropogenic factor in the emergence of fungal pathogens, particularly those of crop plants and, to a lesser extent, of livestock, is the unique cradle for pathogen evolution provided by agriculture. Agro-ecosystems are characterized by huge populations of genetically uniform individuals. A few varieties of one crop may constitute almost all of the host variability throughout particular climatic regions, or even worldwide (11, 78, 95). In contrast to the patchy distribution of hosts in natural ecosystems, large cropping areas mean that pathogen populations rarely experience bottlenecks or loss of diversity (87). Pathogen populations are enormous, and coinfection by multiple strains is the norm, increasing the rates of mutation, HGT, and hybridization (67). Similarly, intensive farming practices, with multiple crops per year, reduce the temporal diversity of host availability that is a feature of natural ecosystems. Moreover, fungal pathogens have no need for resting spores if the host is cultivated year-round and instead undergo extra cycles of asexual propagation and clonal spread, bolstering population sizes still further (76, 95). For example, the crop-destroying rice blast fungus *M. oryzae* is almost entirely clonal, while its wild relatives rely on sexual reproduction to produce the resting spores needed for persistence in natural ecosystems (11, 69). Similar processes are argued to have led to the extremely rapid emergence of *P. destructans* and WNS across North America, because bats disperse asexual conidia along their migratory routes (96).

Examples of the importance of agro-ecosystem uniformity in shaping the evolution of agricultural pathogens can be seen today in many economically important fungal crop pathogens. *Z. tritici*, for example, diverged from its nearest relatives around the time that wheat was domesticated and has its center of diversity in the same location—the Fertile Crescent—as wheat itself (78). The wild relatives of *Z. tritici* have a wider host range, which was lost in the genetic bottleneck that followed the initial jump by the ancestors of *Z. tritici* onto wheat. However, as wheat cultivation spread around the world, the pathogen went global too; there are signatures in its genome indicating rapid population growth and diversification (78, 95).

Similarly, many of the pathogens discussed in "Current Examples of Emerging Infectious Fungal Diseases" benefit from these features of agro-ecosystems. As seen in "Natural Factors Promoting the Emergence of New Invasive Fungal Diseases," these invasive, pathogenic fungi share the ability to establish year-round populations through infection of a range of hosts including wild species (21, 40, 81, 91, 97, 98), allowing spread through agricultural and unmanaged ecosystems. Such pathogens often have long-distance dispersal ability, bolstering genetic diversity during establishment (12, 21, 66, 87). Further, many are accidentally propagated on volunteer host plants, which appear between growing seasons, along roadsides or in other anthropogenic environments (1, 23, 24, 41, 66) (see Fig. 3).

Apart from crop plants, there is only one organism whose pathogens show such rapid evolution and propensity for host shifts, host jumps, and HGT events. This is because the density and uniformity of the agro-ecosystem, and high levels of pathogen pollution within it, are matched by only one species: the species supported by the agro-ecosystems—ourselves. With a huge, dense population, *Homo sapiens* has provided its pathogens with a similar evolutionary cradle. This means that lessons may be learned in interdisciplinary research into the evolution and epidemiology of crop and human pathogens (78).

A final anthropogenic factor influencing the emergence of invasive pathogens is climate change. This has both direct and indirect effects on the spread of fungi. One study found that pests and pathogens of crops are moving poleward as global temperatures increase, with fungi leading the charge (99, 100). Limitations imposed by cold winter temperatures, both on fungi themselves and on their hosts, are gradually lifting at higher latitudes, while perhaps, higher summer temperatures impose restrictions closer to the equator. This process is exemplified by the increasing severity of outbreaks of chytridiomycosis across amphibian populations in the high Pyrenees as the onset of spring arrives earlier in the year (101).

In addition to these direct effects, climate change influences agriculture, with crops increasingly grown outside of their natural ranges. With the spread of crop species comes pathogen pollution and all its attendant risks, including enemy release for pathogens which are noncompetitive in the hosts' home range. In addition, hosts will be exposed to pathogens native to the new location, which may adapt to them and eventually then spread into their original range (11, 78).

Panama disease as a case study concerning anthropogenic disease spread

Panama disease, also called *Fusarium* wilt, is an aggressive disease of banana first recorded in Australia in 1876. By 1950, it had spread throughout banana growing regions worldwide (102). Its spread was facilitated by the global monoculture of Gros Michel banana, the only variety grown for export at the time (102). Banana, a seedless triploid (three copies of every genome) resulting from hybridization of two *Musa* species, is propagated via rhizomes (103). Like infected seed (see "Anthropogenic Factors Promoting the Emergence of New Invasive Fungal Diseases"), symptomless but infected rhizomes can be transported around the world, carrying the disease with them (102). The disease can be soilborne, with microconidia entering roots and traveling through the vascular system, followed by further microconidation, allowing spread throughout the plant (104). Chlamydospores (thick-walled spores) survive in soil for up to 30 years. Gros Michel banana was first infected with this disease in Panama, providing its common name; the causal agent was identified in Cuba in 1910 and named *F. oxysporum* f. sp. *cubense* (*Foc*) (103). *F. oxysporum* causes vascular wilt disease in a wide range of plants, although individual *formae speciales* often show a restricted host range (see "Natural Factors Promoting the Emergence of New Invasive Fungal Diseases"). Within *Foc* are four races. Race 1 caused exceptional losses in Gros Michel plantations. Race 2 infects various varieties of cooking banana, whose fruits are not grown for export, and race 3 infects a different host species altogether (104). Abandoning Gros Michel, commercial growers turned to a new variety, Cavendish, which existed as a curiosity in collections in the United Kingdom and Honduras. Cavendish has appropriate qualities for export and resists *Foc* race 1 (103). Thus, trade in the world's favorite fruit was saved—for a time. In the late 1960s, reports appeared of a similar wilt disease infecting Cavendish banana, which in 1994, was confirmed as a new race of *Foc* (104). Race 4 also infects plantains, so it threatens two important crops. Initially, *Foc* Race 4 was confined to the Eastern hemisphere; its discovery in Jordan in 2014 (104) means that it is now likely to spread through Africa and, in time, will probably reach the banana plantations of the Americas. Treatment is problematic, because *Foc* is highly resistant to fungicide. A new, resistant banana variety will be needed if drastic losses in production are to be prevented.

THREATS TO NATURAL ECOSYSTEMS FROM EMERGING FUNGI

We have already seen that fungal diseases can have huge impacts on tree species, for example, in Dutch elm disease and chestnut blight. "Changing Landscapes: Chestnut Blight and Dutch Elm Disease" described the effect of the devastation of the Appalachian chestnut on biodiversity, as well as changes to landscapes and the resulting cultural impact. A similar example, which has received extensive attention, is the case of ash dieback in the United Kingdom and Europe. Ash dieback is a fatal disease of ash (*Fraxinus excelsior*). It is caused by *H. fraxineus* (previously *Chalara fraxinea*), an ascomycete native to Asia which is thought to have spread into and around Europe on infected but asymptomatic nursery stock (76, 105) (Fig. 6). *H. fraxineus* has devastated ash woodland throughout Europe, with up to

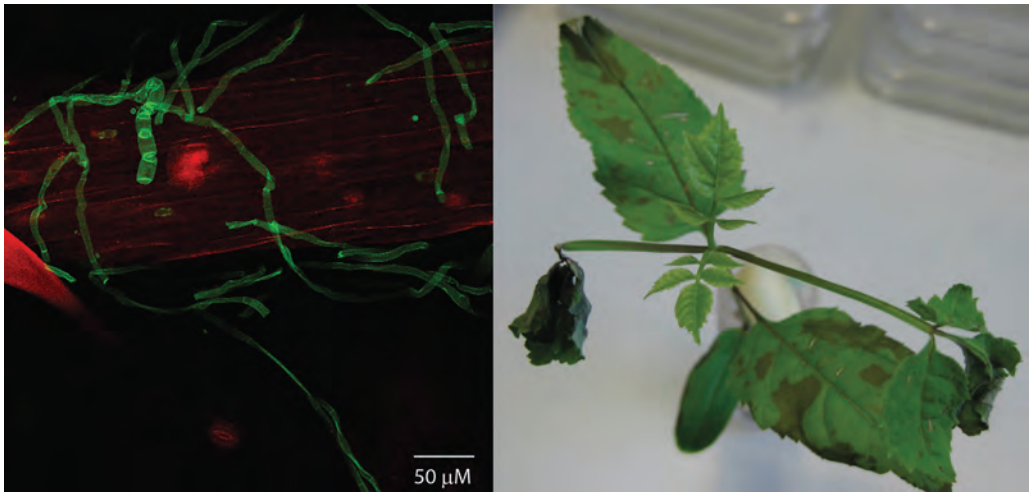


Figure 6 *Hymenoscyphus fraxineus* on ash. Left, hyphae (green; stained with fluorescein isothiocyanate-labeled wheat-germ agglutinin) of *H. fraxineus*, visualized by confocal microscopy, growing over and out of the vascular tissue of an ash leaf (red; stained with propidium iodide). Right, ash die-back symptoms on an ash seedling under laboratory conditions. (Photos: Helen Fones.)

100% of trees affected and with ash now considered a threatened species in several countries (106). This disease attracted public and scientific interest in the United Kingdom, where ash is common in woodlands and hedgerows. Studies indicate that around 45 species are dependent on ash, among a total of around 450 species associated with this tree (107). Ash is a pioneer of and keystone species in European riparian forests, as well as being a hedgerow plant and an urban amenity species (106).

H. fraxineus displays very high levels of outcrossing, resulting in rapid gene flow between populations (108–110). This means that even among emerging fungal diseases, *H. fraxineus* has high evolutionary potential. New genotypes with increased virulence or pathogenicity, whether arising by mutation, introgression, or recombination, quickly spread throughout European populations of this fungus. *H. fraxineus* shows much greater genetic diversity in Asia than in Europe, and Asian strains show higher virulence and pathogenicity to ash (111). With global trade and transport, it is only a matter of time until this destructive potential reaches Europe. Further, there are known to be various sympatric (species inhabiting the same geographic region) *Hymenoscyphus* species (112, 113) with which hybridization may ensue. As well as possible evolution of increased virulence, the possibility of host shifts onto additional *Fraxinus* or *Oleaceae* species, or even host jumps onto other trees entirely, is therefore high for this pathogen (113, 114, 128).

Another keystone species affected by an emerging fungal pathogen is the Hawaiian ʻŌhiʻa tree (*Metrosideros polymorpha*). This tree is threatened by the new disease rapid ʻŌhiʻa death, caused by the fungus *Ceratocystis fimbriata*. In this disease, the fungus infects the sapwood, causing death within weeks. Mortality rates are high: about 50% of infected trees die. Transmission may be by infected soil, cuttings, insect vectors, or windblown insect frass (powder produced by boring insects) (115, 116). The disease is of particular concern, because ʻŌhiʻa is a dominant species in the Hawaiian rainforest, determining succession and canopy structure and providing a habitat for many endangered species (115). Moreover, it is a key pioneer in the colonization of lava flows because it has a root system that is particularly able to penetrate cracks and is adapted to withstand toxic volcanic gases (117, 118).

Another ecosystem service provided by large tree species is carbon sequestration. American chestnut was an efficient carbon sink in its native range (119), capable of absorbing up to 35 metric megatons of CO_2 had the trees not been destroyed by *C. parasitica*. Similarly, Dutch elm disease is calculated to have prevented the absorption of a metric megaton of CO_2. These values do not include the vast potential carbon release from decaying wood of the dead trees (93).

Vertebrate biodiversity is an important component of food webs, and the losses of neotropical amphibian species have caused demonstrable changes in ecosystem functioning. This is compounded by the fact that

amphibians are both important grazers in their aquatic tadpole forms as well as insect-predators in their terrestrial adult morphs. In Central America, chytridiomycosis has changed patterns of amphibian biodiversity. Here, community composition across the region was severely disrupted by epidemics at multiple sites, and many of the unique species of these communities were eliminated, with disproportionate effects on endemic species. This resulted in a homogenization of the regional fauna and ecological homogenization of reproductive mode and habitat (120). Alongside the losses of biodiversity, economic consequences are starting to be attributed to emerging fungal diseases of vertebrates. While correctly attributing the economic cost of ecosystem functions is still in its infancy, valuations have estimated the losses to U.S. agriculture that are the result of declines in bat populations at more than $3.7 billion per year (121). Indeed, there are many ecosystem services which are hard to quantify but may be threatened or diminished when species are decimated by emerging fungal diseases. These include prevention of flooding, provision of clean air and water, pollination of crops and other plants, as well as the cultural, religious, and recreational significance of host species to humans. Where biodiversity is threatened, we are also at risk of losing potential pharmaceuticals and various other genetic resources which could be used to improve domestic species.

THREATS TO AGRICULTURE FROM EMERGING FUNGI

As well as their devastating effects on natural ecosystems and wild species, emerging fungal pathogens pose serious threats to agricultural production and food security. This is evident from "Current Examples of Emerging Infectious Fungal Diseases," where we saw the huge losses that can result from particular fungal pathogens. A recent, detailed study estimated that in the three major wheat growing countries of the European Union (United Kingdom, France, and Germany) losses caused by the fungus *Z. tritici* (Septoria leaf blotch of wheat) surpass €1,400 million yearly. This is despite deployment of the most resistant wheat varieties and application of fungicides at a further cost of over €900 million per growing season (70). These losses are due to just one disease, in conditions optimized for its control. It is clear that losses worldwide to fungal pathogens impact significantly on productivity and costs. Affordable food security will therefore depend on our ability to mitigate this threat.

Unfortunately, that ability is constantly challenged by the pathogens themselves. As we have discussed, emerging fungal pathogens are exceptionally adaptable. As well as increasing the risk that they will spread to new hosts and new places, this adaptability allows pathogens to repeatedly break the resistance provided by elite crop varieties. At least one wheat variety previously resistant to *Z. tritici* has failed due to new virulence acquisition by the pathogen (122); resistance breeding is complicated by linkage between *Z. tritici* resistance and low crop yield (123).

Not only do fungi have the ability to out-evolve and overcome the defenses of resistant hosts, but they can evolve or acquire genes for fungicide resistance, as we saw in "Azole-Resistant Aspergillosis." As such, these strains overcome our main mechanisms for protecting crops from disease and thus ensuring food security. The very traits that promote their becoming invasive pathogens also increase the risk of their developing fungicide resistance. For example, few curative fungicides exist for *Z. tritici* in wheat; there is reliance on azoles and strobilurins, to which the fungus is beginning to show resistance (70). Indeed, azole-resistant fungi are becoming increasingly common, a problem partly attributed to the persistence of azoles in soil (124). This increases incidental contact with fungi, creating selection pressure for resistance, which may be conferred by a single gene such as *Aspergillus nidulans atrB*, encoding a multidrug transporter conferring resistance to azoles and other fungicides (125). This is concerning not just because of the implications for agriculture, but also because many of these fungi or their close relatives cause mycoses in humans. Examples include *A. fumigatus*, the cause of invasive aspergillosis, a potentially fatal disease generally treated with triazole fungicides (see "Azole-Resistant Aspergillosis"). A single amino acid change and a 34-bp tandem repeat in the promoter region of the *Cyp51A* gene encoding the target protein are sufficient to provide resistance to various frontline triazoles that are used to treat severe human infection (61, 124).

Similarly, the generalist fungus *M. phaseolina* (see "Current Examples of Emerging Infectious Fungal Diseases"), which attacks immunocompromised patients, is treated with azole fungicides, but azole-resistant fungal strains are appearing (33). Given the environmental persistence of azoles and the high evolutionary potential of crossover pathogens such as *M. phaseolina* (126), it may be necessary to curb the agricultural use of azoles to maintain their medical efficacy, as discussed in "Azole-Resistant Aspergillosis." However, there are not yet effective replacements for azoles in agriculture.

CONCLUSIONS

Fungal infections have wreaked havoc with our harvests through the centuries. Fungi continue to cause widespread damage in crops. Recently, however, we have witnessed their growing impact on ecosystem resilience and on human health: there has been a dramatic decline in populations of amphibians and bats and an increased incidence of fungal infections in humans. Human activity and global climate change are intensifying fungal dispersal by modifying natural ecosystems and creating new opportunities for evolution. We must take steps to mitigate the risk of these infectious diseases spreading globally, for without action, fungal infections will lead to reduced crop yields, damaging food security. Increased agricultural reliance on fungicides will promote the evolution of fungicide resistance, which trait may then spread into pathogens of wild species and of ourselves. Biodiversity and human health will then suffer alongside agriculture, increasing the risk to ecosystems and ecosystem services at a time when pressures to exploit land to feed humans will be heightened. We must therefore ensure that research on the various host, pathogen, and environment interactions is fueled by adequate research funding and that disease detection is improved and then used to impose stricter global biosecurity protocols in the international trade of plant and animal species.

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Fungi and the Human Host

VII

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Fungi that Infect Humans

39

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INTRODUCTION

In its short history of a century and a half, of which the last half-century brought the most dramatic advances, scientific medicine has found ways to cure or to treat millions of ill people who 200 years ago would have died early. Paradoxically, these successes of modern medicine have given rise to large groups of people at risk for fungal infections. Life-saving treatments may now breach normal immune functions, or susceptible patients such as premature newborns now survive long enough to become infected by a fungus. Invasive fungal infections have been very rare over most of our species' history (1), and the fungi that infect healthy humans are a small, if fascinating, group. Many more invasive fungal infections now occur in patients with an underlying serious illness.

Yet at the same time, less than 1/10,000 of the ~5 million fungal species estimated to currently inhabit the earth (2, 3) can infect and cause disease in a human being. The pathogenic fungi are not a monophyletic group, and the capacity for virulence appears to have emerged several times independently in evolutionary history (4). This article intends to illuminate the features by which some fungal species can infect humans and the evolutionary relationships that contributed to their development.

Fungi moved from the oceans onto land in a symbiotic relationship with plants (5–8) and maintain their most intricate host relationships with the plant kingdom. These comprise a spectrum from mutualistic symbiosis to biotrophy (acquiring nutrients from host tissues with specialized structures while downmodulating host immunity), necrotrophy (killing host tissues to feed from them), and saprotrophy (feeding from dead host tissues) (9–12). Among fungi incidentally infecting humans, plant necrotrophs such as *Fusarium* and *Alternaria* and saprotrophs such as *Aspergillus* and *Scedosporium* are common (13, 14). In contrast, plant mutualists such as mycorrhizal fungi and biotrophs such as *Erysiphe* and *Puccinia* are extremely rare agents of human infection, suggesting that an evolutionary strategy of lysing host tissue completely—dead or alive—can target hosts across two kingdoms of life.

Coevolution of animals with fungi shaped animal immune systems. In sponges, which are among the earliest metazoans, a cell surface protein recognizing (1,3)-β-D-glucan has been identified, whose ligand binding activates a tyrosine kinase pathway (15). (1,3)-β-D-Glucan, an abundant fungal cell wall component, thus acts as a fungal pathogen-associated molecular pattern in the earliest animals, demonstrating that defense against fungal attack was a requirement at the dawn of the metazoan era. Evolutionary latecomers such as humans had hundreds of millions of years to hone innate and adaptive immune systems to successfully keep potential fungal invaders in check.

Resistance to fungal infection increased during animal evolution. Insects possess the Toll pathway, by which fungal pattern-recognition receptors ultimately induce antimicrobial peptides, a pathway also used in human innate immunity to respond to fungal invaders (16). As a consequence, insects resist fungal infection more effectively than the early metazoans, yet they still succumb to fungi specialized to prey upon them (17). Infection with ~1,000 species of fungi across five clades kills insects (18).

Vertebrates added the adaptive immune system to their antifungal armamentarium, honing the capacity to

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distinguish self from nonself entities with the major histocompatibility complex, to respond quickly to invaders using memory and recall, and to iteratively increase affinity of soluble and cell-based receptor molecules for pathogens. Consequently, in fish, the first phylum to develop adaptive immunity, invasive fungal diseases due to, e.g., plant necrotrophs and saprophytes *Fusarium, Exophiala*, or *Paecilomyces* (19) are rare (20). Exceptions occur in situations of captivity in which fungal inocula may be overwhelming (21). The importance of the adaptive immune system in protecting humans from fungal infections is underscored by the diseases suffered by humans with defective adaptive immunity: cryptococcal meningoencephalitis, pneumocystis pneumonia, mucosal candidiasis, and massive dermatophyte skin and nail infections afflict individuals who are unable to mount adaptive immune responses.

Humans, endothermic mammals with highly sophisticated innate and adaptive immune systems, are naturally resistant to most invasive fungal infections. None but a small minority of fungi meet the four criteria required to establish invasive human infection: (i) the ability to grow at or above 37°C, (ii) the ability to reach internal tissues by penetrating or circumventing host barriers, e.g., through small airborne cells that directly enter air-filled spaces of lungs and sinuses, (iii) the ability to digest and absorb components of human tissues, and (iv) the ability to withstand the human immune system.

The stringency of these four criteria is demonstrated by the fact that an entire fungal lineage, the Chytridiomycota, is incapable of infecting humans. Chytridiomycota are an ancient lineage that live in watery environments and have preserved the flagellum of the basal fungi (22). These fungi are mainly saprotrophs and plant parasites (23), but their ability to infect animals is demonstrated by the ongoing decimation of amphibians. A chytrid species, *Batrachochytrium dendrobatidis*, has caused precipitous declines in frog populations around the world and is the cause of extinction of over 100 amphibian species (24). A distinct chytrid clade, the *Neocallimastigales*, demonstrates that chytrids can adapt to mammalian body temperatures by forming mutualistic relationships with ruminant mammals such as cattle and deer to which the fungi provide enzymes for digestion of cellulose (23, 25). But most chytrids grow only at cooler temperatures, and none are known to infect mammals.

The fungi that fulfill the four criteria to infect humans (thermal resistance, locomotion through or around host barriers, lysis and absorption of human tissue, and resistance to immune defenses) thus belong to only four lineages: the Zygomycota, Entomophthorales, Ascomycota, and Basidiomycota. They will be discussed in the following sections according to these phylogenetic categories. In addition, they will be divided by the clinically relevant category of primary versus opportunistic pathogens: have they evolved to confront healthy humans' immune systems, or do they infect only immunocompromised humans? While this distinction cannot be sharply drawn, in many cases it reflects an evolutionary history, because for important fungal opportunistic pathogens such as *Fusarium* and *Aspergillus*, humans are only an incidental host, accidentally encountered in their quest for live or dead plant tissue on which to grow.

Casadevall and colleagues have hypothesized that the stable elevated temperature of endothermic (warm-blooded) animals may have been one of the most potent developments in antifungal immunity (1, 26, 27). Protection from invasion by most fungi seems to be a major benefit of the energy expenditure on endothermy (control of body temperature by active balancing of metabolic heat production with heat loss) that drove its convergent evolution in at least two vertebrate lineages: birds and mammals (1).

Casadevall and colleagues proposed that evolution of endothermy and homeothermy produced a "thermal exclusionary zone" in which warmer hosts restrict fungal growth (1). These workers examined the temperature tolerance of over 4,800 fungal isolates of the Dutch national collection of fungi, which included 144 genera among the Dikarya (1). They found that most isolates grew well between 12 and 30°C, but with every 1° increase in temperature >30°C, around 6% fewer strains could grow (1). Calculations of the energetic cost versus infection-avoidance benefit of endothermic physiology arrived at an approximate temperature of many endothermic animals, ~36.5°C, as providing the most favorable balance (26). In fact, mammals have been proposed to have emerged as the dominant large animals at the end of the cretaceous period by selection through a "fungal filter" that prevented a second reptilian age (28), which could explain their remarkable resistance to fungal disease relative to other animals.

These considerations are supported by observations of "behavioral fever" among fungus-infected insects, which as mentioned, are susceptible to numerous fungi. Field observations and controlled experiments show that fungus-infected insects seek external heat sources and can survive longer when they are able to warm themselves (29, 30). Human fever is an even more energy-costly output of tumor necrosis factor-alpha signaling, which is potently induced by invading fungi, as shown for *Candida albicans* by several groups

(31, 32). The persistent fever response to invasive fungal infections is such a sensitive (if completely nonspecific) clinical marker that for over 3 decades since the studies of Pizzo and coworkers (33), it has been used as the sole indication for adding empiric antifungal to antibacterial therapy in neutropenic patients. Studies of the antifungal effect of human fever are lacking, but the general practice of aggressive antipyresis for fungus-infected patients may be questioned (34).

A capacity for locomotion to and through the human body is the second criterion for a fungus to infect a human. In the oceans, fungi originated as unicellular, oval cells propelled by flagella. Having lost their flagella, extant human fungal pathogens must use more indirect modes of movement to enter and spread through the body. Adhesion molecules are virulence factors related to fungal motion strategies because once a fungal cell has reached a favorable location, it needs to be able to stay at the site (35). Morphogenesis is another important virulence factor related to fungal movement. Yeast can readily disseminate to distant sites in the liquid environment of the bloodstream and extracellular fluid. Another advantage of a yeast form is its resistance to mechanical stress, such that most fungi encountered in deep-sea habitats, where hydrostatic pressures are in the range of 146 to 388 atmospheres, are yeast (36). Furthermore, yeasts are small enough to be readily ingested and spread by insects (37) and to become airborne in a desiccated state and act as infectious propagules (38). Primary fungal pathogens, once in the human body, proliferate almost exclusively in their yeast form.

Growth as a round or oval cell was a fundamental fungal trait. Lücking et al., in their comparison of fossil and molecular dating systems of fungal evolution, state that "all dating estimates show that the evolution of filamentous fungi occurred much later than the origin of the fungal lineage itself, suggesting that for a long time after their origin fungi were heterotrophic, unicellular, flagellate, aquatic organisms" (39). When fungi colonized land as plant symbionts (6, 39, 40), a growth form with a large surface area became necessary: mycelial fungal plant symbionts are able to absorb and concentrate essential nutrients which in soil are highly dilute, such as nitrogen and phosphate, and receive sugar from the plant in return (41).

Hyphae enable an active form of locomotion, as opposed to yeast or airborne propagules, which rely on passive transport. Growth at the hyphal tip propels the fungus into new territory, whether in the soil or in a human organ. Hyphae need adhesion molecules at their surface for their role in movement, because the growing tip could not move directionally if older hyphal cells were not tethered to their substratum. In *C. albicans*, hyphae possess unique adhesins that contribute to virulence (42–45).

Hyphal morphogenesis contributes to protection of fungi against ingestion and destruction by amoebae and phagocytes, e.g., in *Cryptococcus neoformans* (46). Amoebae are distant relatives of animals, and the basic mechanisms of phagocytosis, i.e., recognition of prey by cell-surface receptors and killing of ingested organisms by reactive oxygen species, are conserved between amoebae and the phagocytes which all metazoans possess (47–49). The opportunistic human pathogen *C. albicans* responds with hyphal growth when yeasts are ingested by macrophages (50), and *Candida* hyphae then induce macrophage pyroptosis (51–53), a form of apoptosis augmenting the immune response (54, 55).

Morphogenesis between two basic cell shapes, round or ovoid and detachable, or long, filamentous, and connected to multicellular mycelia, occurs among essentially all human pathogenic fungi. Airborne locomotion to reach distant substrates has evolved convergently in the form of dispersal cells among three fungal phyla: asexual sporangiospores among the Zygomycota, asexual conidia as well as meiotic ascospores among the Ascomycota, and meiotic basidiospores among the Basidiomycota. The effectiveness of this form of airborne locomotion is reflected in the continent-wide travel of fungal spores (56), their ubiquity out- and inside human habitations (57), and their isolation at stratospheric elevations up to 20 km (58). Few lineages have completely lost a round or a filamentous cell form. A highly successful human-associated fungus, *C. albicans*, is notable for the facility and frequency with which it switches among a broad spectrum of growth forms in the host (59).

Secretion of digestive enzymes appropriate to dissolve host tissue is the first step for a fungus to use the host as a nutritious substrate. *C. albicans*, a successful human commensal and pathogen, possesses 10 secreted acid proteinases known to contribute to virulence (60, 61). Its phospholipases (62, 63) and lipases (64–66) contribute to its pathogenesis. Infection of humans can be achieved only by fungi that secrete hydrolases suitable to digest human tissue, which include primary mammalian pathogens such as *Histoplasma capsulatum* (67, 68) and incidental, opportunistic pathogens such as *Fusarium* spp. (69) or *Aspergillus fumigatus* (70). A recent compelling genomic study comparing human-pathogenic Onygenales with five nonpathogenic, closely related species found that in the pathogens, gene families encoding plant-dissolving enzymes had dwindled, while those encoding hydrolases of animal tissues were enriched (71).

A. fumigatus is an interesting case of a saprotrophic fungus acting as a ferocious pathogen in specific immunocompromised populations. It is neither a plant necrotroph nor an amoeban or animal parasite. The features which turn *A. fumigatus* from a helpful recycler of plant carbon and nitrogen when it proliferates in our compost piles into a killer of patients with phagocyte defects, appear to be its high thermotolerance and its profuse production of hydrolases (72). These features lend it an edge among the microbial competition in decaying plant matter and by apparent coincidence enable it to withstand the human fever response and to rapidly lyse tissue in the absence of defending neutrophils when it happens to land in human lungs.

After hydrolysis of host macromolecules, possession of transporters to absorb the released oligo- and monomers of nitrogen, carbon, and phosphate sources, as well as micronutrients, is required of a human fungal pathogen. Acquisition and toxicity management of metallic micronutrients such as iron and zinc are especially critical because of their role as cofactors in enzymatic processes such as respiration and superoxide dismutation.

The host's withholding of these elements from bacterial pathogens has been termed "nutritional immunity" (73), and the same mechanisms limit growth of pathogenic fungi. For example, the human host actively sequesters iron during infection to withhold it from the pathogen (74–78). Conversely, pathogenic fungi such as *Candida*, *Histoplasma*, and *Paracoccidioides* deploy specific tools to wrest iron from the host (79–82). Interestingly, *A. fumigatus* utilizes siderophores, high-affinity iron chelators, to acquire environmental iron but has no uptake systems for host-specific iron sources such as heme and ferritin (83), consistent with its lifestyle as a plant saprobe and an only accidental pathogen of humans. The importance of iron sequestration in the host to defend against fungal growth was dramatically illustrated by an increased incidence of disseminated Mucorales infections in patients receiving the iron chelator deferoxamine (84).

In the micronutrient competition between pathogen and host, copper is a unique two-edged sword: while required for virulence of human fungal pathogens such as *Cryptococcus*, *Candida*, and *Paracoccidioides* (85–89), copper is also concentrated by activated host macrophages for lysosomal generation of reactive oxygen species to kill the ingested pathogen (90). Detoxification of copper has been shown to be required for virulence of *C. neoformans* (91, 92). Copper is a cofactor for a critical enzyme defending pathogens against reactive oxygen species, superoxide dismutase, and in a clever maneuver, *C. albicans* preempts rising ambient copper concentrations deployed by the host during infection: it decreases copper transporter expression and maintains activity of its superoxide dismutase by utilizing an alternative cofactor, manganese (93).

As Dzik emphasized in her superb review of the past half billion years' evolution of immunity (48), phagocytosis is a basal ability of unicellular eukaryotes such as amoebae and of the mobile cells of all metazoans. Macrophages, like amoebae, use cell surface receptors to recognize their prey (47) and reactive oxygen species to kill it (94). Fungal parasites of amoebae and of animals honed their strategies to evade these mechanisms over millions of years, and fungi that infect healthy humans devote a large portion of their physiology to evading or withstanding the immune system. Their strategies sometimes differ even between lineages of a single species.

The effectiveness of the human immune system against fungi can be judged by comparing the few fungal species that infect healthy humans with the hundreds of species that have been isolated from infections of immunocompromised patients. Conversely, the most effective fungal human parasites, as judged by the wideness of their distribution and the numbers of hosts they inhabit, are able to downmodulate human immune responses, thereby decreasing the risk to themselves that they will be immunologically eliminated. Additionally, unless a human-specialized fungal pathogen has a mode of transportation back from the soil to the next human host (as the Ajellomycetaceae do), killing the host is a dead end for the fungus, and the evolutionary winners manage to moderate host inflammation and disease. Among the pathogens that adopt these strategies are *Pneumocystis jirovecii* (Fig. 1A,B), the agent of pneumocystis pneumonia, the anthropophilic dermatophytes that cause ringworm and toenail fungus, and possibly the *Candida* colonizers of human mouths and guts which only invade systemically when normal defenses are breached (Fig. 1C).

Fungal cells use complex networks of sensing and signaling systems to fulfill these four criteria of pathogenicity. Experimental disruption of just one component leads to loss of virulence, including mechanosensor, calcineurin, protein kinase A, mitogen-activated protein kinase, target of rapamycin (TOR), and high-osmolarity signaling pathways (95–106).

FUNGI CAPABLE OF INFECTING HEALTHY HUMANS

Most of the fungi capable of infecting healthy humans evolved to infect other hosts such as amoebae, insects,

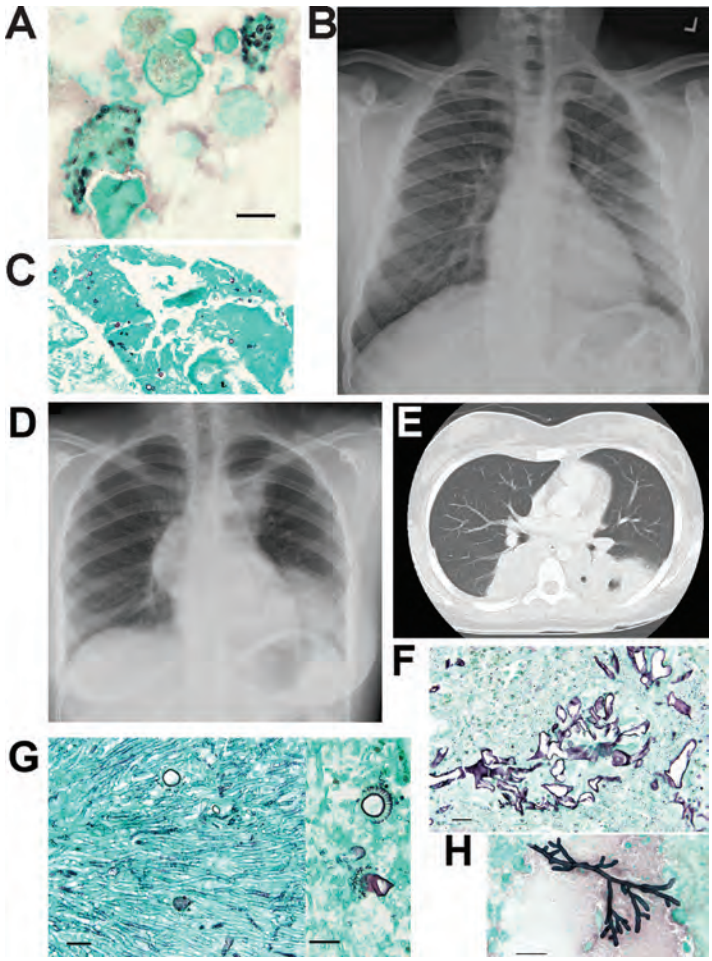


Figure 1 (**A**) Clusters of *Pneumocystis jirovecii* cells in brochoalveolar lavage fluid of an 8-year-old boy, post-lung transplant. Gomori methenamine silver (GMS) stain. Scale bar, 20 μm. (**B**) Chest film of a 17-year-old boy living with HIV, with pneumonia due to *Pneumocystis jirovecii*. (**C**) Biopsy of skin nodule of a 9-year-old boy with aplastic anemia, containing numerous *Candida tropicalis* yeasts. GMS stain. (**D,E**) Posterior-anterior chest film (D) and axial computed tomography scan (E) of a 16-year-old previously healthy girl with cavitating cryptococcal pneumonia. (**F**) *Rhizopus* sp. hyphae in bowel wall of an adolescent boy, post-liver transplant. GMS stain. Scale bar, 20 μm. (**G**) *Aspergillus fumigatus* in maxillary sinusitis and osteomyelitis of a young man, post-intestinal transplant. In the human host, aspergilli produce conidiophores (prominently visible in the image on the right) only in air-filled spaces. GMS stain. Scale bars, 20 μm. (**H**) *A. fumigatus* in bronchoalveolar lavage fluid of a 3-week-old neonate with adenosine deaminase deficiency. Scale bar, 20 μm. (All micrographs: Dr. Harry P. W. Kozakewich, Department of Pathology, Boston Children's Hospital; Radiographs: Department of Radiology, Boston Children's Hospital.)

or small mammals. This is in contrast to bacteria such as *Streptococcus pyogenes* or *Mycobacterium tuberculosis*, whose virulence attributes directly target humans as their primary host. The ability to utilize healthy human hosts arose independently multiple times among three major fungal phyla: the Entomophthoromycota (107), the Ascomycota, and the Basidiomycota.

Entomophthoromycota

Entomophthoromycota are insect pathogens which have attracted the interest of mycologists since the 19th century because of their ability to decimate pests. They have been found as agents of invasive human infection only in subtropical and tropical regions despite their worldwide distribution. The species of genera

pathogenic for humans, *Basidiobolus* and *Conidiobolus*, are evolutionarily distant from each other as well as from the Mucorales, with whom they traditionally were classified in Zygomycota (107).

While the hyphae of Entomophthorales appear to be similar to those of the Mucorales, being broad, ribbony, with right-angle branches and sparse to no septa, their behavior is fundamentally different, in that they do not invade blood vessels (108). Thus, infarction and necrosis are not usual features, unlike in Mucorales infections. Their lytic enzymes, including elastases, collagenases, and lipases, kill arthropods (109–113) and are likely also to digest human tissue (114).

Conidiobolus spp. produce large ballistospores of 30 to 38 μm (115), which are forcibly launched up to 30 cm from their sporangiophores and presumably infect humans when they are inhaled and deposited on the mucosa of nasal air passages (116). Two species, *Conidiobolus coronatus* and *Conidiobolus incongruus*, have been isolated from central facial disease. Gradual progression causes swelling of submucosal and subcutaneous tissue, disfigurement, breathing difficulties, and chronic bacterial sinusitis due to blockage of ostia. Unlike the Mucorales that cause sinus disease, *Conidiobolus* does not usually penetrate into the central nervous system (CNS) (108).

Basidiobolus ranarum causes subcutaneous disease primarily in children in tropical and subtropical Africa, Asia, and the Americas. The fungus can be attached to plant matter in feces of amphibians and reptiles that ingested *Basidiobolus*-infected insects (117) and can be accidentally inoculated under the skin, e.g., by thorns. Nodules appear at inoculation sites (117), sometimes resembling malignancies (118). *Basidiobolus* can also cause gastrointestinal disease, presumably after ingestion, mimicking inflammatory bowel disease or cancer (119–121). Its inability to invade deep organs is likely related to limited thermotolerance: 37°C may be its maximal growth temperature (117).

Neutrophils, eosinophils, and histiocytes respond to the fungal invasion, forming ordered cellular arrays, granulomas, to contain the fungus. Dense eosinophilic material surrounding the hyphae, the Splendore-Hoeppli phenomenon, which is thought to consist of antigen-antibody precipitate (115), is a classical histologic finding.

Ascomycota

Subcutaneous infections of healthy hosts, through traumatic inoculation of fungal elements, e.g., on thorns, is also initiated by a few dozen members of the Ascomycota, which cause chromoblastomycosis and mycetoma of the extremities and trunk. These neglected tropical diseases cause much suffering but only very rarely directly cause death, and they afflict poor people in rural areas, which may explain the limited research attention they have enjoyed and the as yet limited understanding of their pathogenesis.

Chromoblastomycosis is caused by several genera of filamentous pigmented plant saprotrophs, including *Fonsecaea* and *Cladophialophora* and, more rarely, *Phialophora, Rhinocladiella*, and *Exophiala* (122). These fungi convert to growth as chambered round structures called "muriform cells" in the host, which are pathognomonic for the disease. Their melanin-coated cell walls are thought to protect against reactive oxygen species (123). As the superficial infection extends in depth and breadth, verrucous and nodular lesions develop which become intensely pruritic. Chronic inflammation gives rise to lymphedema and squamous cell carcinomas (122). The incidence of chromoblastosis in Brazil is estimated at 3/100,000 (124) and in Madagascar at 14/100,000 (122), though accurate counts are not available. Treatment is difficult and requires months to years of antifungals, of which the most commonly used have been itraconazole and terbinafine (122).

Mycetoma is a destructive subcutaneous infection that spreads by extension to bone and vital organs including the lungs. Amputation of the affected limb is in many cases the final common pathway of treatment courses that failed because of drug toxicity (e.g., in the case of ketoconazole), inability to purchase the years' worth of drugs required, difficulty adhering to such long treatment courses, and poor efficacy of the drugs (125). The syndrome is characterized by nodule formation, sinus tracts, and discharge of macroscopic granules which consist of masses of the pathogenic agent. While actinomycetes including *Nocardia* spp. cause similar clinical syndromes, the disease is often caused by fungi and then termed "eumycetoma." Fungi involved in this infection include *Pseudallescheria boydii*, a feared opportunistic pathogen of neutropenic patients in other contexts, *Fusarium* spp., discussed below, and most commonly, *Madurella mycetomatis*, a member of the Sordariales (126). This melanin-producing species typically does not produce conidiophores in culture, contributing to difficulties in precise identification except when molecular diagnostics are available (127).

Mycetoma occurs around the globe in regions between 30°N and 15°S of the equator, termed the "mycetoma belt," which includes Mexico, Sudan, and India. Risk factors for acquisition of this crippling infection are agricultural work and lack of shoes. A recent genomic study positioning *M. mycetomatis* among Sordariales

species growing in dung of mammals suggests that this agent of mycetoma may reside in dung of domestic animals and enter the subcutaneous space by traumatic inoculation (127). Why only a fraction of the at-risk individuals develop the infection is the subject of studies that have variously implicated polymorphisms in CC chemokine ligand 5 and interleukin-10 promoter regions (128), in the promoter of tissue inhibitor of metalloproteinases (129), and in chitotriosidase, a macrophage enzyme required to break down cell wall chitin of *M. mycetomatis* (130).

More urgent is the question of prevention and treatment: protective clothing, including closed shoes, could do much to avert new mycetoma cases, if at-risk individuals could afford them. Because ketoconazole has unacceptable hepatotoxicity, and itraconazole is only erratically absorbed, a recent finding of remarkably low *in vitro* MICs of ravuconazole for *M. mycetomatis* is highly encouraging, if confirmed clinically, and if this drug or other extended triazoles such as voriconazole, posaconazole, and isavuconazole will be made available to those who need it (131), treatment outcomes might substantially improve. As with most neglected tropical diseases, an improvement of the socioeconomic situation of at-risk people will be the most effective means of prevention.

Pathogenic Onygenales comprise a group of ascomycetous soil dwellers that parasitize mammals and cause systemic infection. Most belong to the family Ajellomycetaceae (132, 133), which includes *Blastomyces dermatitidis* (teleomorph, *Ajellomyces dermatitidis*) *H. capsulatum* (teleomorph, *Ajellomyces capsulatus*), *Paracoccidioides brasiliensis*, *Paracoccidioides lutzii*, and *Lacazia loboi*. *Coccidioides immitis* and *Coccidioides posadasii* in the family Onygenaceae possess similar properties. Ajellomycetaceae are the only phylogenetically related fungal group whose members all cause systemic disease in otherwise healthy humans. As mentioned, phylogenomic analyses show that the pathogenic clades of Onygenales switched from vegetarian to carnivorous lifestyles, losing cellulases and pectinases and gaining proteinases and keratinases (71).

Most genera occur in defined geographic areas, which follow particular soil consistencies, e.g., dry and alkaline for the *Coccidioides* spp. and acidic for *Histoplasma*, and possibly specific host mammals, e.g., rodents for *Coccidioides* (134), bats for *Histoplasma* (135), and armadillo species for *Paracoccidioides* (136, 137). In the soil, they grow as a branched network of hyphal filaments, a mycelium, and utilize dead plant or animal matter for nutrition. Their airborne dispersal cells are produced as conidia from specialized hyphae in *Histoplasma* and *Blastomyces* or as arthrospores from regulated fragmentation of hyphal compartments, in *Coccidioides* and *Paracoccidioides*. *Paracoccidioides* additionally produces aleuroconidia budding directly from hyphae.

Once a mammal inhales conidia or arthrospores, they convert into parasitic yeasts (or spherules in the case of *Coccidioides* spp.) and initiate an infection. The principal signal for their conversion to the yeast or spherule form is mammalian body temperature as their cue to initiate an entire alternative pathogenic growth program. In immunocompromised hosts, the infections are more clinically apparent, more severe, and more likely to disseminate to multiple organs with different organ disease propensities among different genera: *Paracoccidioides* in oral and respiratory mucous membranes (138, 139); *Blastomyces* in bones, joints, and skin (140); *Histoplasma* in multiple organs including the gastrointestinal tract and adrenals, as well as in bones and skin (141); and *Coccidioides* spp. in lungs, skin, deep organs, and in rare worst cases, in the CNS.

Adaptive cellular immunity is required to control the infection with these fungi. For this reason, clinically apparent infections with some members of this group, e.g., *H. capsulatum*, became much more common with the rising prevalence of AIDS. Close relatives within this order pursue very different pathogenic strategies in the same habitat. For example, the teleomorph genus *Ajellomyces* comprises anamorph sister species *H. capsulatum* and *B. dermatitidis*, of which one, *H. capsulatum*, evolved mechanisms to thrive within macrophage phagolysosomes, while the other, *B. dermatitidis*, is a successful extracellular pathogen.

Other thermally dimorphic fungi are rare animal-pathogenic species within orders comprising plant saprobes and pathogens. They are *Sporothrix schenckii* (order Ophiostomatales), which is primarily introduced into the host through skin injuries, and *Talaromyces* (*Penicillium*) *marneffei* (order Eurotiales), which does not infect healthy hosts but due to the AIDS epidemic has become a prevalent and important invasive pathogen in Southeast Asia.

The fact that these evolutionarily distant fungi convert from saprobic hyphal to pathogenic yeast-growth at the temperatures of endothermic animals suggests that an ability to access large nutrient stores of a mammalian host is an advantageous lifestyle option for a soil dweller and is a result of convergent evolution in several phyla. It is also a testament to the suitability of the yeast form for accessing and spreading through a human host.

H. capsulatum hyphae produce macro- and microconidia. The latter are small enough to penetrate into the alveoli of mammalian lungs, where they are phagocytized by alveolar macrophages and convert to yeasts which proliferate in the hostile environment of the phagolysosome. This conversion entails differential transcription of 6 to 20% of the genome (142–144) and is controlled by a network of transcriptional regulators in response to a histidine kinase signal (145–148).

Regulation of translational efficiency of specific transcripts has recently been found to play a role in cell-type interconversions of *H. capsulatum* (149). Differential lengths of upstream leader sequences of cell-type enriched transcripts specify distinct translational activity of the transcripts in the two different cell types, adding a layer of translational regulation to the previously known transcriptional regulation of cell fate. In addition to the transcriptional regulators Ryp2 and Ryp3 that induce yeast-enriched transcripts, expression of a homolog of the *Aspergillus nidulans* WetA conidial regulator is repressed by differential translation in yeast, and its dysregulated expression at 37°C induces inappropriate hyphal growth at mammalian body temperature (149).

Histoplasma thrives in soil enriched with bird and bat droppings. While birds are very rarely infected with *H. capsulatum* (150), bats are frequently infected but respond with minimal inflammation (135), suggesting active repression of the host immune response by the fungus. A broad variety of other infected mammals including humans develop granulomatous inflammation in response to *Histoplasma* yeast (151–153). The fungus may have developed specific mechanisms to repress the host immune response of bats, thereby establishing an efficient amplification cycle by which its mycelial form grows in droppings under bat roosting places, e.g., in caves, and its microconidia are released into the air to be inhaled by the bats that fertilize its soil substrate (135).

Other pathogenic Onygenales, all of which associate with mammals, have equally distinctive ecology and pathobiology. The genus *Coccidioides* comprises the species *C. immitis*, distributed through California into Utah and eastern Washington (154), and *C. posadasii* (155, 156), which is present in Arizona, Texas, Mexico, Central America (157, 158), and South America including Brazil (159), Argentina, Colombia, Paraguay, and Venezuela (160, 161). The human disease was first described by Alejandro Posadas in Argentina, who reported finding large cysts in the widespread skin lesions, which he called "psorospermias," of a cavalry soldier returning from the Chaco (162).

Mycelia of the *Coccidioides* spp. grow ~20 cm below the surface near rodent burrows in hot, arid soils (161, 163). They are thought to remain limited to the surface area in which a mammalian carcass is decaying (164), because they have lost the plant-degrading enzymes such as cellulase and tannase of their ancestral lineages, while acquiring animal tissue-digesting keratinases and metalloproteinases (165). The morphogenetic repertoire of *Coccidioides* is unique in that the parasitic, round form of the fungus in the mammalian host, the spherule, does not bud to the exterior, but produces daughter endospores in the enlarging mother. The maturing spherule protects its progeny from host phagocytic attack through its sheer size of 60 to 100 μm (166), which not even a macrophage of 20 to 80 μm could engulf. The spherule eventually ruptures to release its daughters into host tissue for local replication or hematogenous dissemination.

Coccidioides arthroconidia arise by regulated fragmentation of subsurface hyphae and become airborne when the soil is disturbed by wind or by human or animal activity. Inhalation of arthroconidia begins the infectious process, in which conidia convert to spherules at mammalian body temperatures and begin infecting the lungs. In ~60% of cases, the infection causes no symptoms or a mild respiratory syndrome, but in 40%, febrile pneumonia ensues, among whom a small percentage develop dissemination to the skin, skeletal system, and deep organs including the CNS (167).

Much of our current understanding of *Coccidioides* spp. pathobiology originated with Charles Edward Smith's epidemiologic studies of soldiers stationed in Californian Army airfields during World War II and of 432 patients with erythema nodosum, then considered a hallmark of coccidioidomycosis, in Kern and Tulare Counties of the San Joaquin Valley (168, 169). Smith showed the association of infection with field work such as potato digging and cotton chopping and the absence of human-to-human transmission as well as silent infection in a majority of cases (168, 169). He also demonstrated the role of adaptive immunity in preventing reinfection once the coccidiodin skin test turned positive, i.e., cellular memory had developed, inspiring contemporary vaccine development efforts (168, 170, 171). These findings also explain a possible protective role of long-term low-level exposures, given the increased risk of recent arrivals to endemic areas developing valley fever (168) and given the link with tuberculosis, which can coexist in *Coccidioides* infections and amplify their severity (161).

Coccidioides spp. evolved in North America before formation of the isthmus of Panama connected that

continent with South America ~3 million years ago (172). The genetically homogeneous South American strains, derived from populations in present-day Texas, reached their current distribution 8,940 to 134,000 years ago (172). This timespan coincides with the migration of humans into South America, and it is conceivable that latently infected humans carried *C. posadasii* to their new homes on this continent (173). In addition, other mammals such as migrating bats could contribute to its ongoing spread (174).

Though a mating-competent form has not been observed, meiotic genes are expressed in *Coccidioides* spp. (71, 163), and there is much genetic diversity and little clonality, to the point that within a 10-m^2 area in Tucson, Arizona, multiple genotypes were found (175). Recent hybridization events between the species have been documented: gene flows from *C. posadasii* to *C. immitis*, enriched for genes associated with immune evasion and with cell walls, suggest selective advantages conferred by the introduced genes (176).

Recent sharp increases in human coccidiodomycosis in the United States have been attributed to several factors, including the large numbers of immunologically naive retirees moving to Sunbelt endemic areas and construction in previously undisturbed desert (177). Human incidence and prevalence studies of *Coccidioides* south of the United States are sparse, but exposure studies from Argentina (178) and Mexico suggest a substantial prevalence (179). A concerning factor is the effect of drought and rainfall cycles on *Coccidioides* growth in the soil (180, 181), which may become more pronounced given current climate shifts.

Another unique member of the Ajellomycetaceae, *L. loboi*, a sister genus to *Paracoccidioides*, has been found only in subcutaneous infections of previously healthy humans in the Amazon basin and in dolphins of the Atlantic Ocean and of the Amazon (182, 183). It can (analogously to *M. leprae*) be amplified only in a mouse model but cannot be grown in culture. In contrast, *P. brasiliensis* and *P. lutzii*, prevalent fungal pathogens that cause pneumonia, systemic disease of the monocyte-macrophage system, and destructive lesions of skin and oral mucous membranes (138, 139, 184, 185), occur in Central and South America mainly outside the Amazon region (183).

In Brazil, *Paracoccidioides* was the major cause of deaths from invasive fungal infection between 1996 and 2006 (186). Though millions of people are thought to be infected, only ~2% will develop disease. As in tuberculosis, most disease results from reactivation of the latent pathogen many years after infection, leading to chronic pulmonary forms that can be uni- or multifocal and affect mainly 30- to 50-year-old agricultural workers. Acute or subacute lymphatic forms of paracoccidiosis are less common; they develop in children and young adults weeks to months after infection and are associated with high mortality rates (187). *Paracoccidioides* causes clinical disease in many more men than women, in a ratio up to 13:1, possibly because estrogen blocks the conversion of inhaled arthroconidia to the tissue-invasive yeast form (188). This fungus frequently infects armadillos, and its virulence factors such as the immunodominant adhesin glycoprotein 43 (gp43) (184, 189–191) may have evolved in its coevolution with these ancient mammals (192, 193). Hence, *Paracoccidioides* may be the member of the pathogenic Onygenales (194) to have evolved targeting a mammalian host in South America during that continent's isolation from the other land masses between 130 and 3 million years ago (194, 195).

In experimental systems, gp43 is partially responsible for yeast cell adherence to Vero cells and to extracellular matrix components such as laminin and fibronectin (196). *P. brasiliensis* adheres to and then is taken up by epithelial cells through endocytosis, with the aid of microfilaments and microtubules, triggering host cell apoptosis (196). Proteases subsequently support nutrient acquisition and dissemination of the fungus, e.g., an extracellular thiol-dependent serine protease, which selectively cleaves extracellular matrix-associated proteins *in vitro* and has a unique kind of positive modulation by fungal polysaccharides. Another serine protease (PbSP) interacts with proteins related to folding processes, protein trafficking, and cytoskeleton reorganization (197). A secreted aspartyl protease (Sap) was detected in the fungal cell wall and is highly expressed in a virulent isolate (198).

Some members of the Onygenales infect healthy humans only superficially: the dermatophytes. They cause invasive disease very rarely, e.g., in individuals who cannot induce dectin 1-dependent innate responses to (1,3)-β-D-glucan as a result of homozygous CARD9 mutations (199, 200). Dermatophytes of the family *Arthrodermataceae* specialize in degrading keratins, important structural proteins in vertebrate skin whose extensive cross-linking by disulfide bonds makes them proteinase-resistant (201). Reptilian amniotes had evolved keratin, so the basis for this specialization was available as a fungal substrate 300 million years ago (202).

Analysis of the mitochondrial genomes of six dermatophytes comprising the three morphologically defined anamorphic genera *Trichophyton*, *Microsporum*, and *Epidermophyton*, together with 29 other members of the Ascomycota, confirmed that the dermatophytes are

descended from a common ancestor (are monophyletic) and separated from other fungi only 32 to 50 million years ago (203). Molecular phylogenetic studies have confirmed the grouping of dermatophytes by their ecologic and clinical features, including the distinct sites of the human body they tend to infect (204, 205), but not by their traditional morphology-based taxonomy.

Anthropophilic dermatophytes travel over the world with their human hosts. While many species are endemic to specific geographic regions, some occur worldwide. Their prevalence varies with the lifestyle and socioeconomic conditions of their human hosts and is undergoing continuous epidemiologic changes (206). Unlike their thermally dimorphic Onygenales relatives, they can reach a new host by person-to-person transmission, and their access to new human substrate is made even easier by the ability of their arthroconidia to persist for years in fomites (207). Anthropophilic dermatophytes, relying on person-to-person access to new hosts, can forgo long-distance travel via airborne conidia, which their geophilic relatives produce in larger profusion to reach distant deposits of animal keratin (207).

Coevolution with their animal hosts presumably allowed dermatophytes to evade or resist the evolutionarily more ancient innate immune mechanisms, so that today their control requires adaptive cellular immunity (208). By restricting their usual habitat to the most superficial keratinized layer of the skin and its appendages, dermatophytes reduce their contact with immune cells. Human-specific dermatophytes (anthropophiles) are able to downregulate host inflammation to establish a chronic infection (209, 210). In contrast, humans eliminate soil-dwelling (geophilic) and animal-specific (zoophilic) dermatophytes by a vigorous inflammatory response.

Basidiomycota

Cryptococcus

Cryptococcus (Fig. 1D,E) is a genus containing diverse environmental saprophytes worldwide, among which two species infect humans: *C. neoformans* and *Cryptococcus gattii*. Only in the past few decades have their phylogeny, genetics, cell and sexual biology, and virulence been elucidated, because before the AIDS epidemic they were rare human pathogens (211). Until the international medical community's goal of an AIDS-free generation by 2030 is reached, e.g., through implementation of UNAIDS' 90-90-90 goals (212, 213), cryptococcus will continue to cause lethal infections: for 2006, 957,900 cases of cryptococcal meningoencephalitis associated with AIDS were estimated, resulting in 624,700 deaths (214). Most of these deaths occur in Africa and are caused by *C. neoformans* var. *grubii*. Its sister species, *C. gattii*, has recently emerged in the American Northwest as a cause of pneumonia and other deep organ infections in apparently healthy people and in animals. Hence, in addition to expanding its geographic range (215), the fungus may also be undergoing continued evolution toward increased virulence (216–218).

C. gattii causes invasive disease and death in wild and domestic land mammals, ocean mammals, and birds, as well as pneumonia and meningoencephalitis in previously healthy people (219–223). A wealth of analyses of pathogenic cryptococci over the past several decades have shown that *C. gattii* in many settings is a more common primary pathogen than *C. neoformans* (218, 224), and new reports continue to blur the artificial boundaries between primary and opportunistic pathogens among the *Cryptococcus* species (225–227), illustrating the limited utility of these terms (228). A recent report has indicated that the presence of anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) auto-antibody is a risk factor for *C. gattii* CNS infection in otherwise healthy individuals (229). It suggests that patients with cryptococcal CNS infection considered "immunocompetent" may carry immune defects which cannot yet be identified by routine immunological screening.

C. neoformans and *C. gattii* are believed to have separated ~40 million years ago (230, 231), diverging from saprobic cryptococcal phyla most likely in Africa (230) and subsequently spreading around the world in waves of clonal expansion punctuated by interbreeding events (232). Their broad and flexible sexual repertoire allows for genetic diversification and hybridization, introducing hybrid vigor, as well as an extremely efficient form of inbreeding: same-sex mating (233–235).

The nomenclature of cryptococcal subtypes continues to evolve as more genomic data are analyzed, but presently *C. neoformans* var. *grubii* has at least three distinct genotypes, VNI, VNII, and VNB (236), and *C. gattii* has been organized into four major genotypes, VGI, VGII, VGIII, and VGIV (235), which contain further subtypes such as VGIIa-c. Therefore, there are numerous lineages within each species, as well as hybrids between them, with distinct virulence properties that have been demonstrated worldwide (237). It is important to emphasize that even the standard H99 (*C. neoformans* var. *grubii*) strain can micro-evolve in the laboratory over short periods of time with measurable consequences for its virulence (238).

An origin of *C. neoformans* var. *grubii* in southern Africa is supported by the finding that this is the site of its highest genotypic diversity, and a collection of strains from Botswana contained 12% isolates of the a mating type, which in other sites is extremely rare (236). Enrichment of *C. neoformans* in bird droppings (236) and its ability to mate on pigeon droppings (239) are consistent with the idea that birds may have been one of its vectors of spread; another travel mode, with ocean currents, is suggested by survival of cryptococci for at least a year in fresh- and seawater (240).

C. gattii has a similar focus of unique symmetry of alpha and a cells in South America. While clinical and environmental isolates of the alpha mating type vastly predominate in other parts of the world, and isolates of the a type are almost never encountered, the South American distribution is 0.8 a to 1.0 alpha (241). *C. gattii* has been isolated from dozens of tree species, especially from sites of wood decay and insect consumption of vegetable matter (242–245). Could migrating insects rather than birds have expanded *C. gattii*'s range? In any case, its culture from a tree in the midst of a pristine Amazon rainforest (243) supports the idea of its longstanding presence in South America.

The pathogenic cryptococci enter a human by inhalation of infectious cells: dried yeast or possibly basidiospores, the products of meiosis after mating. When these small airborne cells, <5 μm in size, are inhaled, pneumonia can ensue in susceptible hosts, and upon gaining access to the bloodstream, the yeast can disseminate to all organs including skin. *Cryptococcus* has a special predilection for the CNS and causes subacute meningoencephalitis, in which high intracranial pressure plays an especially deleterious role and which, left untreated, is lethal. The fungus can persist for years in the lung or in sites of previous dissemination and reactivates only upon weakened immune surveillance.

C. neoformans and *C. gattii* have classically been shown to share important virulence traits lacking in their rarely pathogenic sister species: a thick capsule composed of glucurono- and galactoxylomannan which, by analogy to encapsulated bacteria such as *Streptococcus pneumoniae*, blocks phagocytosis of the organism unless it is opsonized (246, 247); cell wall melanin which shields the fungal cell from oxidative and other chemical stress (248, 249); and the ability to grow at endothermic mammals' temperature range (250).

The cryptococcal capsule is a unique organelle with many roles in infection. Capsular glucuronoxylomannan and other macromolecules, as well as virulence-associated proteins, are carried through the cell wall by vesicles that originate from the late endosome (multivesicular body) and are targeted to the extracellular space (251, 252). Upon phagocytosis, large amounts of capsular polysaccharide are shed to fill the phagocytic cells' cytoplasm (253, 254).

Further protective mechanisms against phagocyte attack are disruption of phagolysosome membranes and interference with full acidification of this organelle (253); detoxification of oxidative and nitrosative stressors with neutralizing enzymes such as superoxide dismutase, glutathione reductase, and thioredoxins (255); and a highly sophisticated system to detoxify the copper wielded by host macrophages against the fungus while acquiring sufficient amounts of this critical micronutrient (91, 256).

Cryptococci can promote their own extrusion from the intracellular environment with a process called nonlytic exocytosis (vomocytosis). In this process the vesicle with the yeast and its potentially important extracellular "virulence factors" is expelled from a phagocytic cell without the latter's demise (257). It is apparent that cryptococci can live and move around in both an intracellular and extracellular lifestyle within the host.

Diversification of a population is an important survival mechanism in stress situations like the host interaction. Cryptococci can physically diversify by size in the host, producing large Titan cells and small drop cells. Titan cells are giant cells of up to 50 μm in diameter that are too large to be phagocytosed (258, 259) and in which DNA replication and growth are not followed by mitosis (259). There is some evidence of a dormant cell population in hosts latently infected with *Cryptococcus* (260). Cryptococci further diversify by age, in that senescent cells are more resistant to phagocytosis, oxidative stress, and killing by antifungals than younger cells (261) and are more likely to undergo phenotypic switching (262).

The transcriptome of *Cryptococcus* in the human host demonstrates a stress reaction (263). Furthermore, under *in vivo* stress conditions the genome shows plasticity with changes in genes and chromosome structure (264), which has implications for antifungal drug resistance and its stability (265). Many antiphagocytic mechanisms are controlled by two GATA transcription factors and are likely to involve coordinated regulation of numerous physiologic processes, because hundreds of genes are differentially expressed in their mutants (266, 267). Hypothetically, global restructuring of the transcriptional landscape during the encounter with the host, by positive feedback loops of chromatin modifications uniquely discovered in the fungal kingdom,

may lead to a global "pathogenic" versus "saprobic" chromatin state (268).

How did cryptococci, for which humans are only incidental hosts, evolve their extensive repertoire of virulence factors? As mentioned above, amoebae use essentially the same mechanisms for engulfment and digestion of nonself cells as the phagocytes of metazoans (48). A prolific line of experimentation has shown that cryptococci utilize the same mechanisms against amoebae as against mammalian hosts and put them to use to proliferate in amoebae (253, 269), suggesting coevolution with amoeboid predators in the soil (253).

The recent development of a lateral flow assay for detection of cryptococcal antigen in blood and cerebrospinal fluid has importantly allowed a sensitive, specific, inexpensive and technologically facile test to be used effectively in resource-limited environments for clinical care (270). Clearer understanding of the ecology, epidemiology, and evolution of cryptococci will emerge with the availability of more microbiological capacity in resource-poor countries: cultures of blood and cerebrospinal fluid currently cannot be performed in medical settings where most patients with cryptococcosis seek help.

OPPORTUNISTIC FUNGAL PATHOGENS OF HUMANS

A sufficiently immunocompromised host can be infected by hundreds of environmental fungal species that grow at human core temperatures. However, a predictable set of actors among Mucorales (Fig. 1F), Ascomycota, and Basidiomycota is known to cause the most common invasive infections in immunocompromised individuals, and they will be discussed next according to their phylogenetic affiliations, which predict important parameters of their physiology.

Basidiomycota

Besides the *Cryptococcus* spp. discussed above, other Basidiomycota that grow predominantly as yeast in the host, including *Malassezia* spp., *Trichosporon asahii*, and other members of the human skin microbiota, are opportunists for patients with venous catheters and certain immunosuppressed patients.

Ascomycota

Candida

Candida spp. are among the most common human pathogenic fungi (Fig. 1C), because the majority of the human population carries members of the genus *Candida* on their mucous membranes. The genus consists of approximately 150 species, but the overwhelming majority (>90%) of *Candida* infections are caused by only four species: *C. albicans* (>50% of all cases), *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis*. *C. albicans*, *C. parapsilosis*, and *C. tropicalis* belong to the CTG clade, a phylogenetic group that translates the CTG codon into serine instead of leucine (271), while *C. glabrata*, the second most common species in many studies, is more closely related to the baker's yeast *Saccharomyces cerevisiae*. These *Candida* species are commensals and natural members of the microbiota of mucosal surfaces (*C. albicans*, *C. parapsilosis*, and *C. glabrata*) and the skin (*C. parapsilosis*), and *C. tropicalis* is also an environmental fungus.

Until the 1950s, humans survived only briefly with diseases during which *C. albicans* could significantly invade the host. Invasive candidiasis was very rare (272, 273). Yet paradoxically, *C. albicans* is an efficient invasive pathogen in humans with immune defects, causing mucous membrane infections in individuals with ineffective adaptive cellular immunity and fatal disseminated infections in patients lacking functional innate immune cells (neutrophils). Humans so prevalently acquire adaptive immunity against *Candida* during childhood that for decades, a skin test containing *Candida* antigens was used as an anergy control in tuberculin skin testing for *M. tuberculosis* with the purified protein derivative (274). Given the rarity of invasive candidiasis until very recently, it is unlikely that *C. albicans*' pathogenic repertoire evolved to enable invasive disease. Moreover, unlike the pathogenic Onygenales, which produce airborne conidia from the soil-growing mycelia nourished on lethally infected animals, *Candida* has no mechanism to return to other human hosts once it has caused lethal infection. However, every human community has a population of individuals with temporary deficits of adaptive immunity: infants and young children.

Children are the community reservoirs of numerous human-adapted pathogens before they, naturally or by vaccination, develop specific adaptive immunity. This pervasive principle was recently dramatically illustrated when novel pneumococcal vaccinations for children were implemented, leading to decreased pneumococcal disease of adults over 65 (275). Before they develop anticandidal adaptive immunity, healthy children are susceptible to candidal infection of the oral mucous membranes, popularly termed thrush. We posit that the pathogenic repertoire of *C. albicans*, including biofilm formation and disruption of epithelial barriers, evolved for temporary mucosal infection of infants, which provides the fungus with the opportunity to replicate to

substantial numbers and, via infants' uncontrolled oral secretions, to easily spread to and colonize their adult contacts. While *C. albicans* biofilms at present are most troubling in patients with implanted foreign material including vascular catheters, and negatively impact quality of life in millions of denture wearers, this species' capacity for biofilm formation did not evolve in interactions with man-made plastic materials. A comprehensive study of *C. albicans* transcriptional networks controlling biofilm formation has found that genes upregulated in biofilms arose relatively recently during the evolution of *C. albicans*, after divergence of the CTG clade (276), consistent with the idea that mucous membrane biofilms are an evolutionary hallmark of this species and its close relatives.

Due to the medical relevance of *C. albicans*, the biology, genetics, and pathogenicity mechanisms of this organism have been particularly well studied. In most cases, infections with *C. albicans* are endogenous infections. Therefore, pathogenicity includes a shift from harmless colonization to overgrowth on superficial tissues and invasion into deeper tissue. The major reservoir of *C. albicans* is the human gut, and recent studies have begun to focus on its interaction with bacteria and on the commensal lifestyle, because the evolutionary selection pressure on this fungus has likely been as a commensal organism (277, 278). It has been shown that certain bacterial populations can prevent outgrowth of *C. albicans* and that the presence of *C. albicans* influences community reassembly after treatment with antibiotics (reviewed in 279). Of note, emergence of *C. albicans* and *C. glabrata* has been shown in ≈75% of cases with ultra-low-diversity microbial populations during prolonged stays in intensive care units, providing further indirect evidence for frequent colonization of *Candida* species in the gut (280). Depending on the degree of host immunocompromise, all four human-associated *Candida* species can cause life-threatening disseminated and systemic infections.

While traditionally described as dimorphic, *C. albicans* is in fact a polymorphic fungus and can grow as a spherical yeast, filamentous pseudohyphae or true hyphae, chlamydospores, and in various switching morphologies. All morphologies have a distinct but flexible cell wall structure with key functions in pathogenicity, such as adhesion, invasion and host recognition. Key components are glycoproteins, (1,3)- and (1,6)-β-D-glucan, and chitin.

While the role of chlamydospores remains mysterious, all other *C. albicans* morphologies are associated with distinct biological and pathogenic functions. Switching between opaque and white budding yeast has been linked to mating and biofilm formation; the yeast form is thought to be critical for disseminated infections (281) and spread via the bloodstream (282), and the filamentous form has been shown to be essential for invasion into epithelial cells.

Pathogenicity mechanisms differ between superficial and invasive infections, but in all stages the four fungal virulence properties are required. Initially, attachment to epithelial cells is required and mediated by cell surface adhesins, some of which, such as Hwp1 and Als3, are linked to hyphal morphology (42, 283). Damage of epithelial cells is due to the physical force of hyphal extension, as well as to secreted hydrolytic enzymes and toxins.

Recently, the first peptide toxin of a human-pathogenic fungus was discovered in *C. albicans* (284). This toxin, candidalysin, is a proteolytic product of the strictly hypha-associated polypeptide Ece1. Once hyphae are produced, the *ECE1* gene is expressed and Ece1 is produced, processed by the Golgi proteases Kex2 and Kex1, and secreted. The third of eight peptides in total, candidalysin integrates into host membranes and causes pore-like lesions and damage. Intriguingly, candidalysin is also a key signal for the host to activate the danger response pathway, which has been identified as the signaling pathway that is characteristic for the commensal-to-pathogen shift of *C. albicans* on epithelial surfaces (285).

Adaptation to the different host niches from mucosal surfaces to deeper tissue, blood, and organs is a crucial part of *C. albicans* pathogenicity. This includes metabolic adaptation, and the metabolism of *C. albicans* is known to be very flexible. This is also true for nutrient uptake strategies, in particular, metal acquisition mechanisms (76, 286). Within the several niches and during confrontation with the host immune system, the fungus can adapt to various stresses such as osmolarity, reactive oxygen species, or pH (287). The latter includes not only cellular adaptation, but also active manipulation of the environmental pH (288).

Furthermore, immune evasion strategies to avoid or overcome the immune response of the host are critical. For example, *C. albicans* and *C. glabrata* can survive phagocytosis by macrophages, proliferate intracellularly, and escape (289). Another mechanism of immune evasion and drug resistance strategy is the production of biofilms, which also occurs on medical devices such as catheters.

Although *C. albicans* was originally described as a member of the artificial group of "fungi imperfecti" due to the proposed lack of a sexual cycle, recent studies discovered complete sets of genes required for sexual

reproduction, which led to the discovery of (i) parasexual cycles which permit recombination in the absence of meiosis and (ii) a haploid form of *C. albicans* (290, 291). Much recent work, led by the Berman laboratory, has emphasized the importance of *C. albicans* genomic diversification as its strategy to adapt to shifting environments, including drug exposure. *C. albicans* is able to diversify a population exposed to drugs and other stressors by generating unbalanced chromosomal duplications and deletions (292) and by generating tetra- and aneuploid cells through its parasexual cycle and random chromosome loss (293).

Aspergillus

Aspergillus spp. have distinct abilities to infect humans. Among them, the opportunistic pathogen par excellence *A. fumigatus* initiates infection in air-filled spaces such as the sinuses and lungs (Fig. 1G,H). When unchecked in these sites by phagocytes of innate immunity, its proliferation causes foci of necrotizing pneumonia that may coalesce and destroy the lung. Given its septated hyphae whose compartments can survive fragmentation, it can also hematogenously disseminate to highly perfused organs including the brain, the eyes, and the kidneys. Upon encountering blood vessels, *Aspergillus* hyphae tend to enter and follow their course, clogging the vessel and causing infarction of downstream tissue. This angioinvasive behavior may be attributable to thigmotropism, the ability to sense and follow contours, which aspergilli have in common with fungi of diverse phyla (294, 295).

A. fumigatus causes ~90% of invasive aspergillosis (296), so it will be discussed here for the diverse *Aspergillus* spp. involved in human disease. *A. fumigatus* evolved as a thermophilic plant saprobe. It is well-adapted to the high temperatures that occur during bacterial decomposition of dead plants and tolerates thermophilic bacteria's optimum temperature of around 55°C (297). Clearly, its evolution of virulence factors including secretion of a very broad and redundant range of hydrolases (298, 299) is unrelated to humans because the only significant humans susceptible to invasive aspergillosis before the advent of iatrogenic immunosuppression were those born with mutations in NADPH oxidase, i.e., with chronic granulomatous disease.

Among *A. fumigatus* virulence attributes are pyomelanin and dihydroxynaphthalene melanin, with which it coats its airborne dispersal spores, the conidia (300). Melanin protects conidia from sunlight and oxidative stress in the environment, and in human alveolar macrophages it blocks phagolysosomal acidification (300) and pyroptosis (301). Other secondary metabolites that interfere with phagocyte function are fumagillin, helvolic acid, and gliotoxin (300, 302). Sophisticated biosynthetic pathways for these toxins evolved presumably to defend the fungus against protozoans or small metazoans such as nematodes encountered in its environment. Nevertheless, once conidia germinate and produce a hypha, they are recognized by alveolar macrophages and subsequently destroyed by neutrophils (303–307). Human defenses against aspergilli are very effective: the average person is estimated to inhale between hundreds and tens of thousands of *A. fumigatus* conidia each day without developing infection (308–310).

Non-*fumigatus* aspergilli, *Fusarium*, *Pseudallescheria*, and other opportunistic ascomycetous fungal pathogens

These pathogens cause invasive disease during profound immunosuppression. They were historically divided into hyalohyphomycetes (lacking cell wall pigment) and phaeohyphomycetes (with melanized cell walls), but these distinctions have no taxonomic or prognostic significance. Among the nonpigmented genera are the non-*fumigatus Aspergillus* spp. (311), *Fusarium* spp. and their teleomorph *Nectria* spp. (312), and *P. boydii* and its *Scedosporium* anamorphs (313). *Scedosporium*, a species complex currently being redefined by molecular studies (234), is a saprotroph in watery environments and a cause of devastating pneumonia with occasional dissemination to the CNS after near-drowning incidents. These fungi tend to be resistant to older antifungals, in particular, amphotericin B; newer triazoles are the preferred therapeutic option (314).

Fusarium spp. have evolved to infect plants, and the genomes of certain lineages contain one or more dispensable chromosomes encoding plant pathogenicity factors that may be horizontally transferred within the genus (315). Genes encoding pathogenicity-associated proteins such as secreted hydrolytic enzymes and signaling molecules appear to be evolving at an increased rate compared with other parts of the genomes of three distinct plant-pathogenic *Fusarium* spp., suggesting ongoing coevolution of plant host and fungal pathogen (316).

A recent exquisite study of *Fusarium graminearum* infecting maize stalks (317) identified both plant host-specific and nonspecific induction of virulence factors: early upregulation of plant-directed enzymes such as pectinases, which hydrolyze plant extracellular matrix, as well as induction of genes required for growth during phosphate starvation (317). During early growth between plant cells, *F. graminearum* cells upregulate phosphatases and phosphate transporters and induce synthesis of membrane lipids lacking phosphate (318),

thus freeing up phosphate for ribosome biosynthesis and DNA replication (317). Given the many links between bacterial virulence and phosphate homeostasis (319), these responses of *Fusarium* cells upon encountering a plant host are likely to contribute to virulence in humans as well. In *Fusarium*, morphogenesis additionally contributes to virulence for humans, because yeast-like (adventitious) cells are produced from hyphae in the host (320) and typically spread widely through the bloodstream to cause numerous foci of infection in the skin.

Pigmented filamentous ascomycetes, also called dematiaceous fungi, infect immunocompromised and, rarely, immunocompetent individuals to cause phaeohyphomycosis. Some are also among the agents of chromoblastomycosis. The melanin these fungi elaborate in their cell walls protects them against reactive oxygen species in the environment and in the host. Their pathobiology includes brain abscesses (*Cladophialophora bantianum*, *Ramichloridium* spp., *Dactylaria gallopava*), keratitis, sinus or soft tissue infections, ulcers, and cysts (*Exophiala jeanselmei*, *Exophiala dermatitidis*, *Curvularia*, *Bipolaris*, or *Alternaria* spp.). When directly inoculated into tissue, these fungi such as *Exserohilum rostratum* can cause devastating disease, as evidenced by a tragic meningitis outbreak due to contaminated injectable steroids (321).

Pneumocystis

P. jirovecii belongs to the subphylum Taphrinomycotina, a clade of distinct fungi thought to have diverged at the separation of Ascomycota from Basidiomycota; it is the human-adapted member of the genus *Pneumocystis*, in which each species is able to infect only one specific mammalian host. *Pneumocystis* was first described in 1909 by Carlos Chagas (the discoverer of *Trypanosoma cruzi*, after whom the disease caused by this flagellate parasite is named) in a remarkable 70-page paper on trypanosomiasis published in Portuguese and German, the two languages arranged side-by-side on the page (322). In this paper he described inoculating the blood of three ill children into guinea pigs. After some days, he observed "parasitic forms" in the lungs of guinea pigs from two of the children, which he interpreted as a developmental stage of the flagellate. This lung-residing organism was renamed *Pneumocystis* when husband-and-wife team P. and M. Delanoë, researchers at the Institut Pasteur in Paris, found it in the lungs of local sewer rats that had never been infected with flagellates (323).

The numerous species of the large genus *Pneumocystis* are now known to have coevolved in such close association with their specific mammalian hosts (324) that their mitochondrial rRNA sequences have been used as markers for the phylogeny and phylogeography of the bats and rodents they inhabit (325–327). The human-specific *Pneumocystis* species was renamed *jirovecii* once it was understood to be completely distinct (328) from the rat-specific *P. carinii* identified by the Delanoës in Parisian rats (329–331), with no possibility of host cross-infection (328).

In the 1940s and 1950s, epidemics of "interstitial plasma cell pneumonia" among malnourished infants occurred in Central European countries, particularly Germany, Czechoslovakia, and Austria, which subsided when nutrition improved as the continent recovered from World War II (332). High incidence in orphanages was reported from Iran in the 1960s (333), and sporadic cases among malnourished or immunocompromised children and adults were then also reported in the English literature (332). In 1981, the CDC reported five cases of pneumocystis pneumonia in Los Angeles in the first description of the syndrome that was to be called AIDS (334). Since then, pneumocystis pneumonia incidence has exploded (335)—a devastating disease, occasionally outside the lung, with high mortality among HIV-infected people and among the large and varied immunocompromised patient populations emerging over the past 4 decades.

Into the late 1980s, it was unclear to which kingdom of life *Pneumocystis* belonged: protozoa or fungi? rRNA sequencing resolved the issue (336). But still the unique life cycle of *Pneumocystis* as an obligate biotrophic parasite of mammals (337), seen growing in lungs as amoeboid cells with thin cell walls ("trophic forms") much more abundantly than as oval forms with thick walls ("cystic forms," now known to be asci), poses numerous mysteries, especially because no *in vitro* system of culture has been reproducibly established.

The trophic forms are extracellularly attached to alveolar epithelial cells in so tight an association that interdigitations arise (338), and are generally in a haploid state, but may have to undergo mating and meiosis to proliferate (339). If mating and meiosis are obligatory stages of the *Pneumocystis* life cycle, primary homothallism as indicated by genome structures of *P. carinii* and *P. jirovecii* recently described by Almeida et al. (340) would indeed be an efficient strategy: a haploid cell can then mate with its direct offspring and avoid the trouble of switching mating types or awaiting the appearance of a partner of the opposite mating type (341).

Remarkably, while it remains extracellular, *Pneumocystis* relies on the host for a broad range of metabolic functions and hence has lost a large complement of

metabolic and transporter-encoding genes, as determined in a recent landmark genome analysis (342). It lacks ~80% of the genes for amino acid synthesis that are present in yeast, is not able to assimilate inorganic nitrogen or sulfur, and is missing nearly all genes of the nucleotide salvage pathways (342). In addition, its lipid metabolism is limited, it lacks fatty acid beta-oxidation, and it does not synthesize ergosterol (342). Lack of ergosterol explains its resistance to classical antifungals such as amphotericin B and azoles.

The cysts are thought to be the infective forms, transmitted by the airborne route from an asymptomatically colonized or an ill, infected person to an uninfected one, so that infants acquire the infection from their caretakers (343). Primary infection seems to occur throughout early childhood, with increased prevalence of antibodies in healthy children over time (344), marking the development of acquired immunity whose cellular component is the decisive defense against proliferation of *Pneumocystis* in the human host. Primary infection may not always be asymptomatic, because *Pneumocystis* may cause upper respiratory tract infection in infants (345). It may also contribute to progression of chronic obstructive pulmonary disease in adults (346).

The main defense of *Pneumocystis* against host immunity seems to be a very large family of major surface glycoproteins, of which up to 179 unique genes were found per species (342). It also avoids activating some innate immune responses by forgoing N-linked mannans on the outer chains of its surface proteins and is the only fungal genus not found to encode chitin synthases (342). These strategies seem to be strikingly successful: because *Pneumocystis* has not experienced on an evolutionary timescale a need to respond to host immune defenses such as oxidative stress or nutrient limitation, it has lost genes required in pH sensing, osmotic and oxidative stress responses, cyclic AMP signaling, and the glyoxylate cycle (342).

Prevalence of colonization of immunocompetent humans is thus much higher than was suspected before the advent of detection by PCR. One study showed, e.g., that 62% of people autopsied after dying suddenly of accidents, homicides, and suicides in the streets of Santiago, Chile, were found to carry *Pneumocystis* (347). Apparently, its long coevolution with its mammalian hosts has fine-tuned a "live-and-let-live" relationship in which the fungus does not significantly damage a normal host, and the host provides a deluxe niche for the fungus.

This equilibrium apparently breaks down only when cellular adaptive immunity fails, as in HIV-infected patients not receiving antiretroviral therapy or in patients undergoing iatrogenic immunosuppression. Then the fungus proliferates in the lung and elicits a strong inflammatory response which is the primary cause of failing gas exchange and may require corticosteroid therapy along with direct antipneumocystis treatments to reverse respiratory failure. While its stripped-down metabolic functions allow only for a very slow replication cycle of 5 to 8 days (342, 348), the ineffective host immune response leads to a cycle of fungal replication and futile inflammation that despite appropriate therapy can be fatal.

Mucorales

Mucorales cause invasive disease in specific hosts, among whom the most numerous are diabetics. The number of diabetic adults has more than doubled over 3 decades (349) in a slow-motion explosion of diabetes following adoption of processed, high-glycemic-load foods around the world; disease-controlling medication and equipment are out of reach for millions of poor diabetics (350). Within this development is embedded an unexplained rise of type 1 diabetes incidence of 3 to 4% per year (351), which with its potential for diabetic ketoacidosis is putting large numbers of people newly at risk for devastating infections with a group of ancient fungi: the Mucorales.

Additionally, Mucorales infect neutropenic and otherwise profoundly immunosuppressed people and patients with elevated serum iron due to hemochromatosis or excessive blood transfusions, as described in the excellent series by Roden at al. (352). The infection begins with entry of airborne sporangiospores into air-filled spaces, i.e., sinuses and lungs. Infections with Mucorales progress rapidly as the fungus grows quickly and relentlessly through tissue planes and blood vessels, causing widespread infarction and necrosis due to its angioinvasive behavior. Angioinvasion is likely due to thigmotropism, the ability to sense and follow the curvature of a surface, which has been experimentally demonstrated for *Mucor mucedo* (294), and distinguishes the Mucorales from its sister clade Entomophthorales, which neither invade blood vessels nor usually cause necrosis. To avoid fatal outcomes (353), management requires three factors: control of underlying disease, surgical debridement, and antifungal therapy with lipid formulations of amphotericin B or, provided the pathogen tests as being susceptible, new extended-spectrum azoles such as posaconazole or isavuconazole (354).

The Mucorales are soil saprotrophs with occasional parasitic behavior toward plants and animals. They comprise an ancient lineage whose evolutionary dis-

tance from the phyla of other human parasitic fungi is apparent in the structure of their hyphae, which are fragile, thin-walled, and lack septa, and in the composition of their cell walls, in which chitin and chitosan play the structural roles that glucans fulfill in the Dikarya (355, 356). Some species such as *Mucor circillenoides* are dimorphic and form yeast in the host (357–359), contributing to dissemination. Spread to distant sites is rare in the monomorphic species because fragments of the coenocytic hyphae, small enough to travel in the bloodstream, are likely to leak cytoplasm and become inviable, but when it occurs, the brain is a common target organ in a usually terminal stage of the infection (353).

Thermotolerance has experimentally been shown to be correlated with virulence among *Rhizopus* species (360) (Fig. 1E). Thermotolerant *Rhizopus microsporum* produces a secreted toxin, rhizoxin, in collaboration with a bacterial endosymbiont, *Burkholderia rhizoxinica* (361). Rhizoxin inhibits mitotic spindle formation in eukaryotes and is known to kill, for example, rice seedlings which then provide nutrients for the fungus as well as for the bacterium. Recent work shows that a *Burkholderia* type 2 secretion system extrudes chitinase to gradually melt the fungal cell wall at sites of bacterial attachment, enabling the *Burkholderia* to gently enter *Rhizopus* hyphae and move to the growing hyphal tip, where they induce spore formation so that each vegetative fungal progeny (sporangiospore) is guaranteed its own bacterial endosymbiont (362, 363). In a meticulous study using murine and *Drosophila* models, a contribution of rhizoxin to *Rhizopus* spp. pathogenesis in animal infection was not found (364). This study suggests that pathogenicity for a human is likely to be an unexpected accident for *Rhizopus* landing in a human alveolus, for which this fungus is evolutionarily unprepared. Conversely, the activity of rhizoxin against human cells (365) suggests a potential for discovery of other endosymbiont-produced toxins of broad specificity that may contribute to Mucorales' production of necrosis.

Another *Rhizopus* species, *R. oryzae*, is responsible for ~70% of human disease (366). One of its important virulence factors is the ability to scavenge iron from the host (367–371). As mentioned, procuring micronutrients such as iron is a basic requirement for a fungal pathogen. In the Mucorales this ability is likely to have evolved in their interaction with bacteria: deferoxamine, a compound used in chelation therapy in patients with iron overload, from which *Rhizopus* notoriously can acquire iron (74), is a bacterial siderophore. The importance of iron homeostasis is also reflected in the recently described genome of a representative of the ancient Mucorales lineage *Lichtheimia*, in which zinc/iron permeases, heme oxygenases and siderophore transporters not present in *Rhizopu*s were discovered, and a link between iron limitation and calcineurin signaling was found (372).

CONCLUSION

Humans are endowed by evolution with robust defenses against invasive fungal disease. Yet our species' social evolution has reached a stage where scientific medicine, in the process of successfully treating many serious diseases, renders their survivors newly vulnerable to invasive fungal infections. We have not yet developed sufficient antifungal diagnostic and treatment modalities to meet the needs of this epidemic, and these are clear tasks for our time. In addition, human social evolution has reached an initial threshold of recognizing that if all lives are equally precious, we must provide available prophylactic, diagnostic, and therapeutic efforts to all who need them. Over the past 20 years, since effective treatment became available, the AIDS pandemic has highlighted this imperative. Monumental efforts of bench science and clinical research synergized to bring an end to AIDS within our reach. The goal set by the United Nations Program on HIV/AIDS (UNAIDS) to end the AIDS pandemic by 2030 is achievable with currently available approaches (373), though its implementation is lagging because sufficient resources are not yet available (374), demonstrating a continued need for advocacy which will ultimately benefit all suffering from treatable infections, or from those that could be treatable if research resources were provided. People suffering from opportunistic as well as from primary invasive fungal infections urgently need resources and research efforts to bring them new diagnostics and treatments regardless of commercial potential. Enormous work over the past 3 decades has opened vast new vistas in fungal biology; we can expand upon them to ultimately fulfill the promises of modern medical advances.

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The Mycobiome: Impact on Health and Disease States

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INTRODUCTION

Research efforts by mycologists over the past few years finally established the fungal community and its previously overlooked members as crucial components of the microbiome. It is now undeniable that the commensal fungal microorganisms, alongside the other components of the microbiota, play a central role in association with the human host. Ongoing research is describing the fungal community and is providing new insights into biological mechanisms by which multidirectional interactions between the microbiome, their genomes (metagenome), metabolites (metabolome), and the human host ultimately affect health and/or disease states.

The term "microbiome" refers to microorganisms (microbiota) and their genomes coexisting with their hosts, including millions of bacteria, fungi, viruses, protozoa, and parasites. "Mycobiome" is a term coined to refer specifically to the fungal component of the microbiome. This microbial consortium starts developing during childbirth as a result of environmental exposures, dietary habits, host genome, and other exogenous and endogenous factors. The microbiome is unique to each individual, much like a fingerprint, and establishes a symbiotic relationship with the human host with various effects on the nutritional, metabolic, immunologic, and physiologic state(s) of the host (1). A disruption of this fine balance leads to imbalance, or "dysbiosis," and has been associated with several disease states.

In a national and international attempt to highlight the role of the microbiome and to unify efforts to describe its role in human health and disease states, the National Institutes of Health and the European Commission launched the Human Microbiome Project to characterize the human microbiota and their genomes (2). Here, we will highlight the role of the fungal community as an indispensable component of the microbiome.

CHARACTERIZATION OF THE HUMAN FUNGAL MICROBIOTA (MYCOBIOME) IN HEALTH STATES

Maintaining a Symbiosis

During the first 3 years of life, starting from birth, the human body codevelops with commensal microorganisms, primarily bacteria and fungi. It is hypothesized that these microbiota and their metabolites interact with the human host in a balanced, or symbiotic, bidirectional manner. The composition of the microbiome in each individual varies among body sites and develops as a result of complex endogenous and exogenous exposures including breast feeding, nutrition, medication exposure, and host genetic factors (3). As much as this healthy host-microbiota interaction is fundamental to the different host body functions and maintaining a "normal" state, any disruption of this balance leads to dysbiosis with contribution to disease states.

Early research efforts in the Human Microbiome Project described the bacteriome in "health states," with more focus on describing the bacteria inhabiting various body sites, in particular the skin, oral cavity, and gastrointestinal tract (the gut). The gastrointestinal microbiome historically attracted the most attention due to its intimate association with the human host and direct impact on the development of the immune system from birth through the secretion of various cyto-

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kines, metabolites, and neurohormones (4). Research describing the role of the bacterial component of the microbiome has established its association with several disease states including but not limited to metabolism, obesity, type 2 diabetes mellitus, atopic dermatitis, allergy, wounds, cystic fibrosis, chronic kidney disease, rheumatoid arthritis, neurodevelopmental disorders, HIV, liver cirrhosis, inflammatory bowel disease, autism, Parkinson's disease, pancreatic cancer, colorectal cancer, esophageal cancer, graft-versus-host disease (GVHD), and others (5–18).

In comparison, relatively little effort has been dedicated to characterizing the mycobiome in health and disease states. It was not until 2010 that serious attempts were initiated to characterize the fungal component of the human microbiome. Accurate description of the mycobiome was made possible by using high-throughput, next-generation sequencing (NGS) approaches (multitag pyrosequencing approach and ion-torrent personal genome medicine) to characterize the oral fungal mycobiome in healthy individuals using pan-fungal internal transcribed spacer primers (19) instead of depending on culture-based techniques.

The Oral Mycobiome

In the first study of its kind, by Ghannoum et al. (19), the investigators used multitag pyrosequencing and internal transcribed spacer primers to analyze oral rinse samples from 20 healthy, nonsmoking adults, and data analysis revealed the presence of 11 nonculturable and 74 culturable (capable of growing on a culture medium) genera. In total, 101 culturable species were detected, and on average each individual was found to have between 9 to 23 species. The core oral mycobiome (COM) and bacteriome were defined as organisms present in ≥20% of investigated subjects. The fungal genera representing the COM of the oral cavity were *Candida* species (75% of individuals), *Cladosporium* species (60%), *Aureobasidium* species (50%), members of the Saccharomycetales (50%), *Aspergillus* species (35%), *Fusarium* species (30%), and *Cryptococcus* species (20%). Interestingly, principle component analysis revealed significant individual mycobiome variations between white and Asian men. Using an advanced NGS approach, this study emphasized (i) the complex nature of the mycobiome that results from an interplay between several genetic and environmental factors and (ii) that one-third of the oral fungi in healthy individuals are not culturable on a culture medium in the laboratory and otherwise could not be identified without the use of high-throughput NGS approaches (19).

The Gastrointestinal Mycobiome

The gastrointestinal mycobiome has been of interest in many studies that characterized the fungal component of the intestinal tract in mice, pigs, rabbits, dogs, and humans in addition to other mammals (20). The growing body of evidence continues to reveal complex and multidirectional interactions between the host gastrointestinal tract and fungi, influencing health and disease states. In healthy individuals, the gastrointestinal mycobiome is thought to include 66 fungal genera, with *Candida* being the predominant one, in addition to 184 species. Some of the most common nonpathogenic genera inhabiting the gastrointestinal tract include *Cladosporium*, *Penicillium*, *Mucor*, and *Saccharomyces*; potentially pathogenic genera include *Aspergillus*, *Candida*, *Cryptococcus*, *Fusarium*, and *Pneumocystis*. In the following sections, we will describe in detail the difference in the mycobiome composition in several gastrointestinal diseases and the possible association between these changes and the development of disease (15, 21–24).

The Skin Mycobiome

It is now established that the human skin microbiome consists of millions of organisms, particularly yeast and dermatophytes. To characterize the "basal" skin mycobiome, Findley et al. (25) sampled 14 skin sites from 10 healthy adults. Interestingly, sample analysis revealed that fungal diversity depends on the body site. *Malassezia* was the most common fungal genus, identified in 11/14 sampled body sites. A high fungal diversity was described in the plantar heel, toenail, and toe web foot sites. Sample analysis from arm sites revealed higher bacterial diversity and lower fungal diversity than core-body (i.e., excluding the arm and foot sites) and foot sites. The plantar heel was the most diverse site, with prevalence of *Malassezia*, *Aspergillus*, *Cryptococcus*, *Rhodotorula*, *Epicoccum*, and others. The investigators described both the bacterial and fungal communities at all of these sites and highlighted a few important points: (i) bacterial and fungal diversities are grouped into clusters, with the same core-body site having similar bacterial and fungal richness; (ii) skin topography and tissue structure are key determinants of the microbiome composition; and (iii) the skin mycobiome is highly variable among body sites.

More recently, Jo et al. (26) compared the skin mycobiomes of healthy children and adults. The child group (age <14 years) had more diverse fungal species, while in the adult group (age range 18 to 39 years), *Malassezia* was the most prevalent fungal species and was identified on the trunk, head, and arm skin sites.

The investigators hypothesized that the fungal communities change in individuals through puberty alongside changes in the skin composition and increases in sebaceous glands. The fact that adults have a more sebaceous skin than children could explain the prevalence of the lipophilic fungi *Malassezia* in this group (26). Leung et al. (27) characterized the skin bacteriome and mycobiome in healthy individuals from Hong Kong and compared them to a Western population, showing that the major fungal species were common among the two cohorts. Nonetheless, they demonstrated that outdoor environments and lifestyles may contribute to the difference in the other mycobiome composition between populations (27).

A STATE OF DYSBIOSIS: ROLE OF THE MYCOBIOME IN DISEASE STATES

Human Immunodeficiency Virus (HIV)

The oral bacteriome and mycobiome in HIV-positive individuals were investigated by Brown et al. and Mukherjee et al. (21, 28). The investigators used high-throughput NGS/multitag pyrosequencing approaches to identify the oral bacteriome and mycobiome in 12 HIV-positive individuals compared with HIV-negative matched controls (28). In the core oral bacteriome, 13 of 14 genera were common between the two cohorts, whereas the COM was different and *Candida* was the most common fungus in both cohorts. When further classifying the *Candida* species, *Candida albicans* was the most prevalent, identified in 58% and 83% of HIV-negative and positive individuals, respectively. In addition, the investigators wanted to further describe any correlation between the oral bacteriome and mycobiome in the two cohorts. By conducting correlation analysis, they were able to identify the presence of 12 and 15 bacterial-fungal pairs in the HIV-positive and HIV-negative cohorts, respectively (28). Furthermore, they identified a nonpathogenic member of the COM, *Pichia*, that exhibits an antagonistic (inhibitory) effect against pathogenic fungi including *Candida*, *Cryptococcus*, *Aspergillus*, and *Fusarium* (28). Adding either *Pichia* cells or the medium they were grown in, referred to as *Pichia* spent medium (PSM), inhibited the growth of *Aspergillus*, *Fusarium*, and *Candida*, in addition to inhibiting *Candida* biofilm formation. Importantly, this study was the first to identify an interaction among different members of the oral mycobiome that was postulated to occur via nutrient limitation or the secretion of a product that modulates growth and interferes with virulence factors.

These *in vitro* results were further tested and validated in an experimental murine model of oral candidiasis (28). When compared with the untreated group, mice treated with PSM had a statistically significant lower fungal burden and infection score. When the investigators examined the tongues of PSM-treated mice, they identified an intact epithelium and few hyphae, whereas nystatin and control (saline-treated) mice had epithelial disruption and extensive fungal growth (28). The inhibitory effect of PSM was proteinaceous in nature and not a secretory metabolite. This is an especially important finding because it identifies a potential novel antifungal agent and a new approach for the management of fungal infections.

The same group conducted a subsequent study using a systems biology approach to correlate the bacteriome, mycobiome, and metabolome data between HIV-positive and -negative individuals (21). Correlation difference network analysis showed that *Rothia*, *Candida*, and certain metabolites, namely phenylacetate, sorbitol, and histamine, demonstrate a statistically significant correlation difference between HIV-positive and -negative cohorts. This study highlights the importance of conducting more studies to further investigate the role of cyclic mono- and dipeptides as key metabolites in quorum sensing or cross-talk between oral bacterial and fungal genera and their functional implications in HIV and other patient populations.

Hepatitis B Virus (HBV)

The microbiome composition of individuals infected with HBV has been described to include both the bacterial and fungal communities. Chen et al. (29) studied the gastrointestinal mycobiome in 161 HBV-positive individuals and healthy volunteers using culture-dependent and -independent (18S rDNA gene amplification-based PCR) techniques. The 161 participants in this study were divided into 4 groups: group 1, HBV cirrhosis patients (n = 38); group 2, chronic HBV patients (n = 35); group 3, HBV carriers (n = 33); and group 4, healthy controls (n = 55). The culture-dependent method identified *Saccharomyces cerevisiae* and four *Candida* species (*C. albicans*, *Candida glabrata*, *Candida krusei*, and *Candida tropicalis*). The culture-independent PCR-based method identified 37 operational taxonomic units, which are clusters of nearly identical sequence tags or phylotypes with a predefined similarity threshold (usually 97%), commonly used to define microbial taxa. Thirty-one different fungal operational taxonomic units were identified, including *Cryptococcus*, *Saccharomyces*, *Penicillium*, and *Galactomyces* species. The investigators identified a higher

abundance of *Candida* and *Saccharomyces* with increased severity of HBV infection, and patients with HBV-cirrhosis or chronic HBV infection had greater fungal diversity than HBV carriers and healthy controls. The results of this study were similar to previous findings linking increased HBV disease severity with an increase in fungal burden and proposing an immune-meditated mechanism. One mechanism proposed by Thomas et al. (30) is related to the deficiency or dysfunction in the mannose binding protein (MBP) during HBV infection. Mannose binding protein is a pattern recognition receptor that binds to fungal cell walls and triggers a host immune response against fungal pathogens. Persistent HBV infection has been linked to mutation in the mannose binding protein leading to an attenuated immune activation and increased colonization by fungal pathogens. Further research is required to confirm the underlying mechanisms.

Inflammatory Bowel Disease

The gastrointestinal microbiome has attracted a lot of researchers in an attempt to identify an association between its bacterial and fungal communities and various diseases. The mycobiome has been described in healthy individuals (31–34), and alternations or symbiosis of its fungal components have been implicated in several gastrointestinal disorders, including peptic ulcer disease (35), irritable bowel syndrome (36), antibiotic-associated diarrhea (37), chemotherapy-induced gastrointestinal mucositis (38), and inflammatory bowel disease (IBD) (39).

IBD refers to a group of inflammatory disorders that affect various anatomic locations of the gastrointestinal tract. IBD includes two major disorders: Crohn's disease (CD), which can affect any part of the gastrointestinal tract from the mouth to the anus, and ulcerative colitis (UC), which primarily affects the colon and the rectum (40–42). Although the pathogenesis of these disorders is not completely understood, they have been related to immune dysfunction in a genetically susceptible host. In fact, gastrointestinal microbiota have been implicated to possibly trigger this inflammatory immune response. The initial discovery of anti-*S. cerevisiae* antibodies in the sera of patients with CD triggered further interest in studying the possible association between various components of the mycobiome and IBD.

Using an 18S rRNA PCR-based method, Ott et al. (39) described the mycobiome present in the fecal samples and intestinal mucosa of patients with CD and UC and healthy controls. The fungal profile was significantly different between the fecal and mucosal samples across the three groups. Nevertheless, Ascomycota and Basidiomycota constituted the majority of the fungal taxa identified in the fecal samples across the three groups, accounting for 92.3% and 7.7% of the recovered fungi, respectively.

Standaert-Vitse et al. studied patients with familial CD and noted a higher colonization rate by *C. albicans* in this population (43). To compare the *Candida* profile in the upper gastrointestinal tract (oral cavity) and lower gastrointestinal tract in patients with IBD, Trojanowska et al. (44) studied samples obtained from patients with UC (n = 72), CD (n = 18) and healthy controls (n = 36). The samples were obtained from throat swabs, colonic aspirates, and biopsies, as well as fecal samples, and analyzed using PCR-based and conventional culture-based methods which favor the growth of predominant fungi. The investigators reported a similar alteration in the profile of non-*albicans Candida* species in both the UC and CD groups. Whereas healthy controls were colonized by *C. tropicalis*, IBD patients had a higher diversity of *Candida* and other species, namely *C. tropicalis*, *C. glabrata*, *C. krusei*, *Candida guilliermondii*, *Candida kefyr*, and *Geotrichium candidum* (44). The results of these experiments highlight that (i) *C. albicans* strains isolated from the oral cavity and different sections share a similar genotype, implicating fungal transmission from the upper to the lower gastrointestinal tract, and (ii) the mycobiome of the oral cavity can act as a future diagnostic marker for the development of IBD. *C. albicans* appears to be more common among patients with CD (43).

Jawhara et al. (45) used experimental murine models of CD and induced colonic inflammation using dextran sodium sulfate. The investigators showed that colonic inflammation promotes *C. albicans* colonization (45) and triggers an immune response leading to the production of antibodies against *C. albicans* and *S. cerevisiae* antigens. After these findings, Iliev et al. (20) performed studies using dextran sodium sulfate to chemically induce inflammation in an experimental murine model deficient in dectin-1, a key receptor encoded by *Clec7a* that mediates the biological effects of fungal (1, 3) β-D-glucans. Compared to wild-type mice, dectin-1-deficient mice had more severe IBD symptoms, histologic alterations, production of proinflammatory cytokines, and weight loss (20). The mycobiome profile of dectin-1-deficient mice was characterized by an increase in the prevalence of pathogenic fungi, *Candida*, and *Trichosporon* accompanied by an increase in inflamed intestinal tissue and a decrease in the nonpathogenic fungi *Saccharomyces* (20). In contrast, the bacterial profile was noted to be similar in the wild-type and dectin-1-deficient mice, suggesting that the mycobiome rather than the bacteriome is a key con-

tributor to initiation of inflammation and pathogenesis of IBD (20). The investigators were also able to show that *C. tropicalis* exacerbates colitis severity in dectin-1-deficient mice, as demonstrated by more weight loss and pathologic evidence of crypt loss and inflammatory cell infiltration (20). However, the mycobiome alone is not sufficient to trigger inflammation or reduce disease severity. Transplanting fecal samples from the wild-type to dectin-1-deficient mice did not affect the severity of IBD, suggesting a more complex mechanism for the development of IBD that includes other host-related factors.

More recently, Hoarau et al. (46) characterized the bacteriome and mycobiome of families with CD, their nondiseased first-degree relatives, and healthy non-CD unrelated volunteers from the same geographic area in northern France-Belgium. In this study, the investigators used next-generation ion-torrent sequencing techniques. The microbiome diversity and abundance analysis revealed a distinct microbiome profile in the CD and nondiseased first-degree relative samples compared to the non-CD unrelated samples (46). Interestingly, the CD and nondiseased first-degree relative groups clustered together and had similar mycobiome but not bacteriome profiles. Additionally, microbial interaction analysis using single and mixed-species biofilms revealed a higher abundance of *Serratia marcescens* and *Escherichia coli* in the CD group as well as *C. tropicalis*. The positive correlation between the three organisms suggests that they interact and form a triple-species biofilm, which was further confirmed by the study investigators (46) and illustrated in Fig. 1. These findings are supported by other studies showing that CD family members have a more similar microbiome profile compared to unrelated healthy volunteers (47, 48). This important paper was the first to identify key intra- and interkingdom microbial interactions and associations that are implicated in disease states (46). The proposed hypothesis for such interkingdom microbial interactions is that the mixed-species biofilms represent a cooperative evolutionary strategy for these organisms to survive the host's immune surveillance (49). As such, the fungal components develop virulence factors including extracellular enzymes and hyphae, while bacteria find shelter in the fungal matrix, escaping antibiotics and immune attack (49).

Based on the knowledge that patients with IBD have a higher abundance of and colonization with *C. albicans*, several studies tested the efficiency of antifungals to manipulate the gut microbiome and decrease disease severity. In one study by Zwolinska-Wcislo et al., treatment with fluconazole or probiotic decreased the severity of UC (50). Samuel et al. retrospectively observed that four of six patients with active IBD who were treated for histoplasmosis with itraconazole achieved clinical and endoscopic remission at the end of the treatment duration (51). These data provide evidence that the fungal community plays an important role in IBD and that approaches to control this component of the microbiota through the use of antifungal and/or probiotics may be a very promising option to control IBD.

ROLE OF THE MYCOBIOME IN CANCER

Given that several environmental, genetic, and dietary factors have been implicated in the initiation and progression of carcinogenesis, it is no surprise that the host microbiome and metabolome have recently been at the forefront as potential modulators of cancer metabolism and targets for cancer therapies. To date, the vast majority, if not all, of the literature investigating the role of the microbiome in carcinogenesis and cancer therapy has focused on the bacterial component. We have growing evidence that the microbiome is implicated in colorectal (18, 52, 53), esophageal (54), and pancreatic cancer (17, 55). In addition, some investigators have focused on the association between certain bacteria and the host's response to chemoimmunotherapy (56–58) and checkpoint inhibitors such as programmed death-ligand 1 (59–61). To our knowledge, there is no dedicated research on the implication of the mycobiome in the initiation and/or progression of carcinogenesis. Further investigations are warranted to describe the fungal profile in different cancers in an attempt to start understanding the role of fungi and their metabolites on carcinogenesis and response to cancer chemoimmunotherapies.

ROLE OF THE MYCOBIOME IN HEMATOPOIETIC CELL TRANSPLANTATION (HCT)

Despite all the advances in the treatment of hematological malignancies, HCT continues to be an effective treatment modality and sometimes the only chance for cure. However, these procedures are associated with significant toxicities that increase morbidity and mortality. The major toxicities related to HCT include opportunistic infections, gastrointestinal toxicities, and GVHD.

Scientists have been investigating the role of the microbiome in HCT outcomes for more than 40 years. Several studies have established an association between the microbiota and HCT outcomes including GVHD, infections, transplant-related mortality, relapse rates,

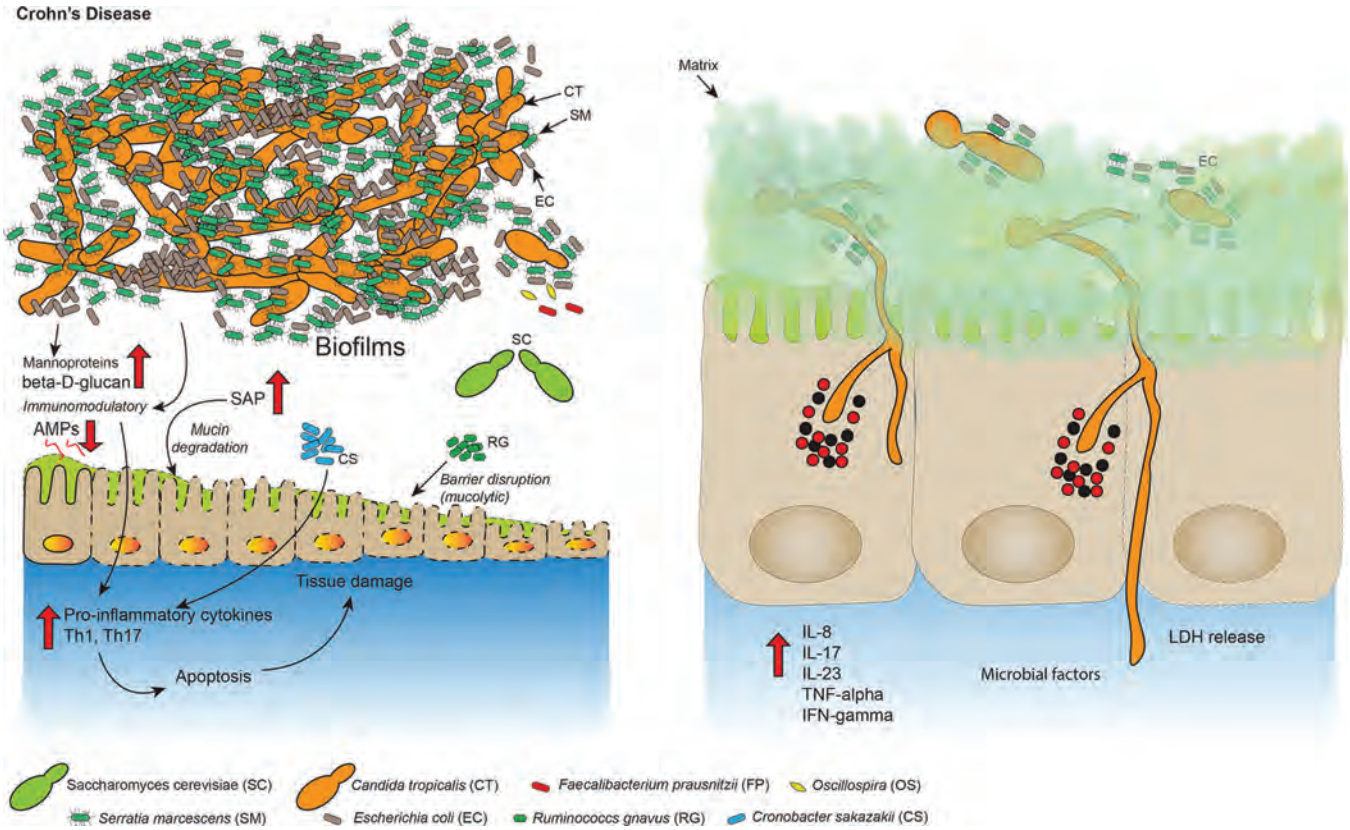


Figure 1 Schema showing potential interactions between bacteria, fungi, and the host in the gut in Crohn's disease (CD). Inter- and intrakingdom interactions impact the host immune system in the setting of CD, resulting in increased levels of proinflammatory cytokines (e.g., Th17 cytokines) under the influence of enteric pathogens and immunomodulatory components of biofilms (e.g., fungal β-glucans, bacterial lipopolysaccharides), causing increased oxidative damage and apoptotic cell death. Additionally, microbial-induced production of mucolytic enzymes may lead to barrier dysfunction, resulting in tissue damage and lesion formation. (**Left**) Interactions between fungi, bacteria, and the host in the gut of CD patients, showing an increase in the levels of *C. tropicalis*, *E. coli*, and *S. marcescens*. (**Right**) Biofilm formation by gut microbiota can influence host response to microbial dysbiosis (71).

and overall survival. Early studies of murine allogeneic HCT models involved gut decontamination in an attempt to prevent gastrointestinal GVHD. However, this strategy fell out of favor in humans due to contradicting results, and it was replaced by the current belief that a more diverse microbiome is associated with better HCT outcomes (62–64). Many studies revealed the role of the microbiome in GVHD (64–68) and mortality post-HCT (62, 69). Similar to oncological malignancies, the majority of the studies have focused on the role of the bacterial rather than the fungal component of the microbiome in HCT. In this article we will highlight the literature focusing on the fungal component, or mycobiome, and its potential contribution to GVHD and other transplant outcomes.

GVHD is a result of a complex process of "immune dysregulation" mediated by allogeneic donor T-cells attacking normal recipient cells. A group at the Fred Hutchinson Cancer Research Center conducted a randomized controlled trial using either fluconazole or placebo for 75 days post-HCT (70). After 8 years of follow-up, patients who were in the fluconazole arm had a statistically significant lower incidence of invasive candidiasis and candidiasis-related death, better overall survival, and lower incidence of severe GVHD (70). This was among the first studies to clearly demonstrate an association between the mycobiome and GVHD along with other transplant outcomes.

Following experiments conducted in mice by Iliev et al. (20) that demonstrated an important role of

dectin-1 in chemically induced colitis and *Candida* colonization, van der Velden and his colleagues investigated whether dectin-1 function and *Candida* colonization in humans had any implications for gastrointestinal GVHD (67). To achieve this objective, the investigators conducted a retrospective analysis on patients undergoing allogeneic HCT who were evaluated for *Candida* colonization and dectin-1 polymorphism on prospectively collected DNA samples (67). Analysis showed that *Candida*-colonized patients had a statistically significant higher incidence of severe grade II to IV GVHD as well as gastrointestinal GVHD (67). Interestingly, *Candida* colonization was more prevalent in patients who had a single nucleotide polymorphism Y238X loss of function polymorphism in the dectin-1 gene. More research is certainly needed to understand the mechanism by which *Candida* and other members of the mycobiome are associated with increased susceptibility and/or protection from gastrointestinal GVHD and other transplant-related outcomes, as is the case with the bacterial component of the microbiome, which has a firmly established role in the pathogenesis of GVHD.

CONCLUSION AND FUTURE DIRECTIONS

Some researchers coined the term "second genome" to underscore the importance of the microbiota and its collective metagenome on their host's health and/or disease. Regardless of the accuracy of this terminology, the message behind it is that the potential magnitude of the microbiome, both bacteriome and mycobiome, is too large to be overlooked. In recognition of this, the Human Microbiome Project was launched nationally and internationally, unifying efforts to characterize the microbiome and to elucidate the functional role of the microbiota and the mechanism(s) by which these organisms and their metabolites (matabolome) may affect health and disease states. Despite recent recognition, more research is needed to characterize the role of the mycobiome component of the microbiome.

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Skin Fungi from Colonization to Infection

41

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INTRODUCTION

Humans are exceptional among vertebrates in that their living tissue is directly exposed to the outside world. In the absence of protective scales, feathers, or fur, the skin has to be highly effective in defending the organism against a gamut of opportunistic fungi surrounding us. Most (sub)cutaneous infections enter the body by implantation through the skin barrier. On intact skin, two types of fungal expansion are noted: (A) colonization by commensals, i.e., growth enabled by conditions prevailing on the skin surface without degradation of tissue, and (B) infection by superficial pathogens that assimilate epidermal keratin and interact with the cellular immune system. In a response-damage framework (1), all fungi are potentially able to cause disease, as a balance between their natural predilection and the immune status of the host. For this reason we will not attribute a fixed ecological term to each species, but rather describe them as growing in a commensal state (A) or in a pathogenic state (B).

Colonization of human skin by allochthonous (i.e., having their preferred niche outside the human host) commensals is coincidental (Fig. 1A). The conditions provided on the skin resemble some essential factors of their natural niche in the environment. The fungi have a "sloppy fitness space" (2); i.e., as long as these factors are met, the fungi are able to survive in habitats that are otherwise unsuitable. Mechanisms for transmission are lacking and thus the infection is a spillover with negative impact on fitness of the etiologic agent. A typical example is the black yeast *Hortaea werneckii*. The species causes tinea nigra, characterized by blackish patches on excessively salty skin of hyperhydrotic individuals acquired, for example, after a holiday at the beach (3). Coastal waters are the natural habitat of the fungus (4). It is a halophile, its ecology being determined by Hog1 homologues of the mitogen-activated protein kinase of *Saccharomyces cerevisiae* (5), being able to grow even in near-saturated NaCl solutions.

Malassezia species have their natural niche on humans, living on human products, i.e., they are autochthonous commensals (Fig. 1B). Clinically, they are recognizable in several common skin disorders including seborrheic dermatitis as pityriasis versicolor (PV), a common skin disorder associated with hyper- or hypopigmentation caused by *Malassezia* (6–8). The lipid-dependent fungi are also part of the benign residential skin flora of most warm-blooded animals including humans (9).

Dermatophytes cause cutaneous infections. The evolutionary history of dermatophytes is determined by their ability to degrade keratin. In humans, they are highly specialized, infecting almost exclusively nails, hair, and epidermis. Three broad ecological groups can be distinguished (Fig. 1C). Ancestral species are geophilic, having a saprobic life cycle in the soil and digesting remains of vertebrate keratin such as feathers and hair. Human infection is sporadic and decreases the fungus' fitness. Zoophilic species reside in closer association with the mammalian host, being carried in pelts and feathers, mostly without causing inflammatory reactions. Anthropophilic species are the most derived dermatophytes, naturally growing on naked human skin. The fungi have developed an intimate interplay with innate and acquired immunity.

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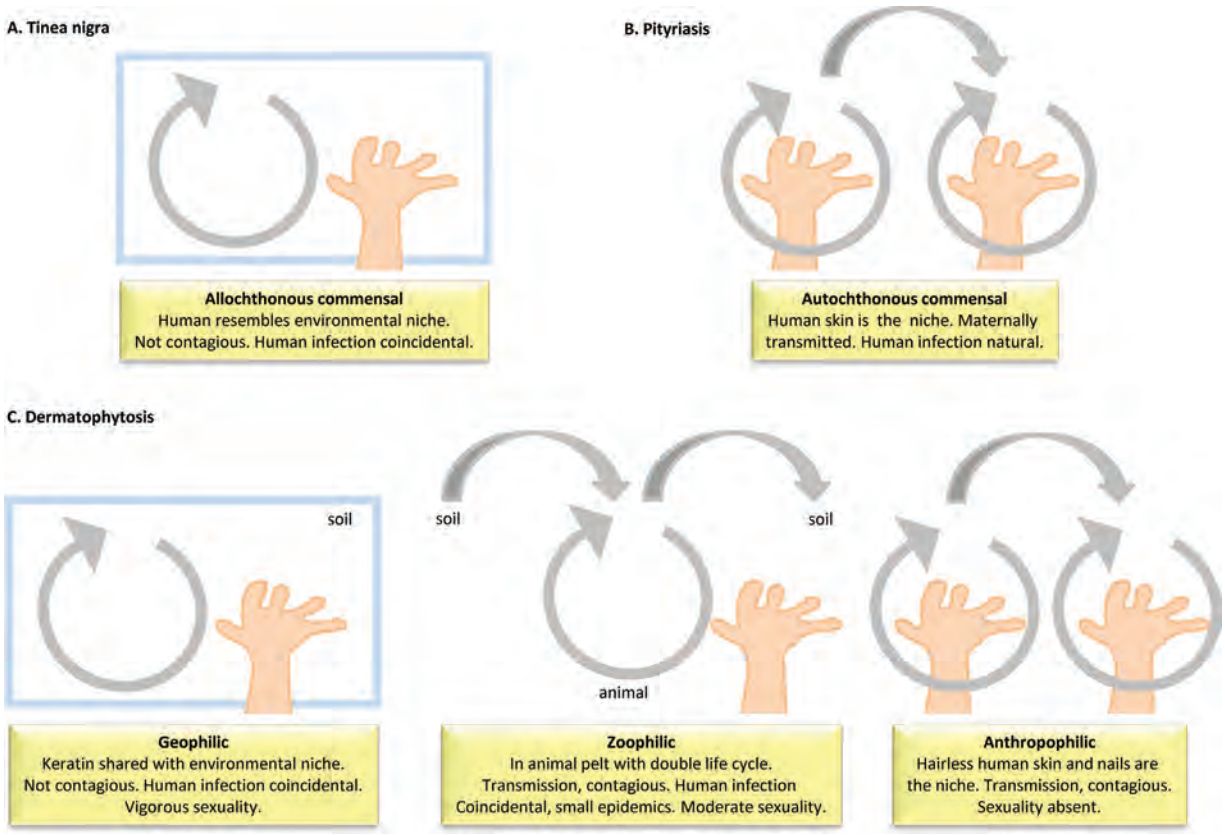


Figure 1 Overview of basic types of fungal occurrence on human skin and modes of transmission.

This review focuses on the natural eukaryotic skin flora: i.e., dermatophytes and *Malassezia*.

DERMATOPHYTES

Biodiversity

Common dermatophytes on humans are classically identified by clinical presentation and macro- and micromorphology. Today, methods of choice to display the total biodiversity and the coherence of species in a phylogenetic system involve multilocus sequencing. Polymorphisms of the rDNA internal transcribed spacer (ITS) region were able to resolve a large number of species (10). The topology of their phylogenetic tree has repeatedly been confirmed by other molecular markers such as *BT2* (11), *TEF1* (12), and *TEF3* (13), and thus the taxonomy of *Arthrodermataceae* seems to have reached stability. In all trees, *Trichophyton* is polyphyletic, containing clinically and ecologically widely different species. Therefore de Hoog et al. (13) confined *Trichophyton* and *Epidermophyton* to mainly anthropophilic species located in derived clusters. Zoophilic species were located at the top and around the middle of the tree and were classified in *Trichophyton*, *Microsporum*, and *Nannizzia*, while the highly diverse geophilic species were located in an ancestral position and were mainly classified in *Arthroderma*, *Lophophyton*, and *Nannizzia* (Fig. 2).

Virulence Factors

Because dermatophytes are almost exclusively localized in keratinized tissues, research on mechanisms of invasion primarily focuses on secreted proteases. All dermatophytes grow well in a medium containing hard keratin as the sole source of carbon and nitrogen. The spectrum of proteases secreted by dermatophytes is similar to that of *Aspergillus*, but differs by multiple endoprotease members of the S8 (subtilisins), M35 (deuterolysin), and M36 (fungalysins) families (14, 15). These belong to endoprotease gene families that have expanded in the *Onygenales*, and to exopeptidases of the M14 family (metallocarboxypeptidases) and M28 (aminopeptidases). At neutral or alkaline pH, dermatophytes secrete two major subtilisins, Sub3 and Sub4,

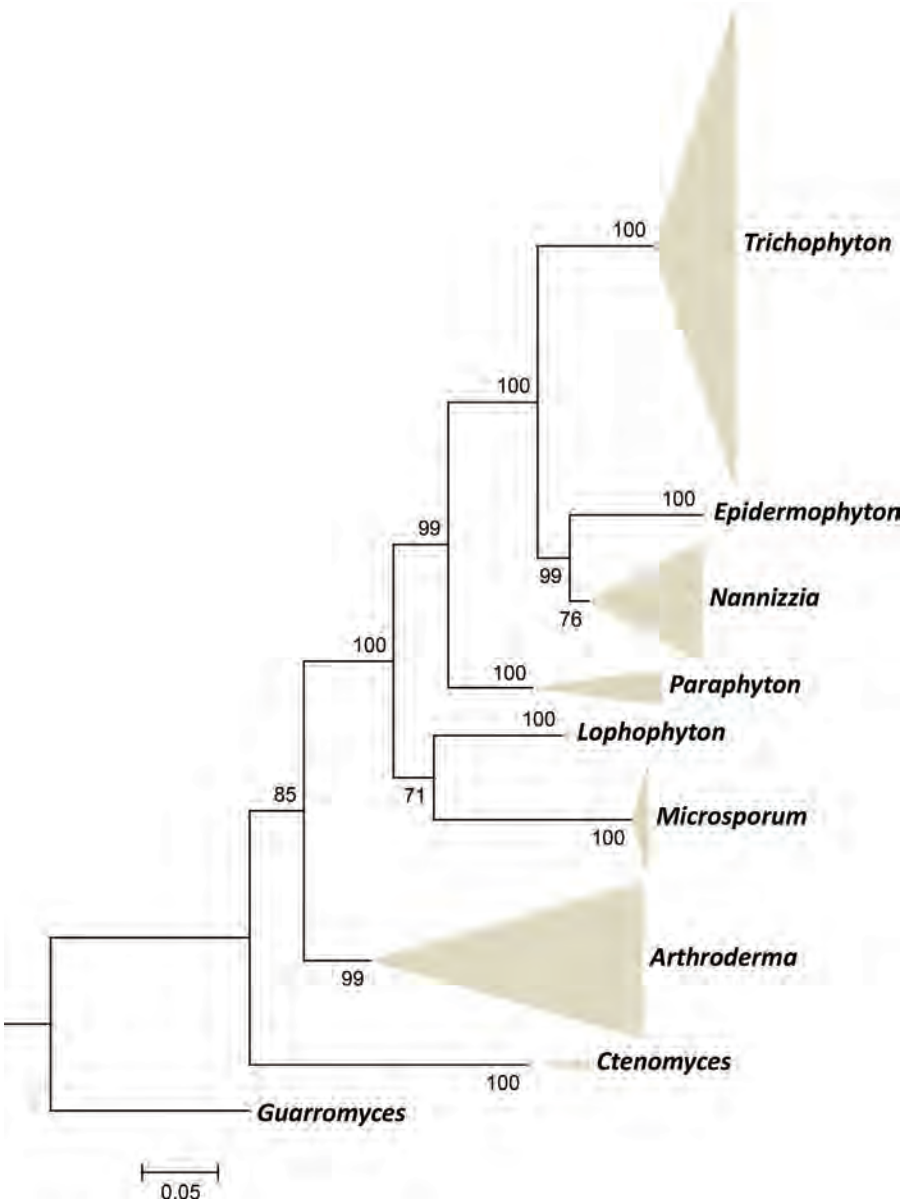


Figure 2 Maximum likelihood phylogenetic tree (RAxML v.8.0.0) based on ITS and partial LSU, TUB, and 60S L10 sequences of *Arthrodermataceae* species using GTRCAT as model, with 1,000 bootstrap replications, shown as collapsed when bootstrap values >70%. *Guarromyces ceretanicus* was selected as outgroup. Reprinted from de Hoog et al. (13).

and two major fungalysins, Mep3 and Mep4, as endopeptidases (14, 16–19). In comparison with proteinase K and subtilisin Carlsberg, *Trichophyton rubrum* secretes subtilisins Sub3 and Sub4, which are more active on keratin azure than on other protein sources such as elastin, suggesting specificity toward hard keratin substrates (17). In addition, dermatophytes secrete various aminopeptidases, e.g., leucine aminopeptidases (Lap1 and Lap2) and dipeptidyl-peptidases (DppIV and DppV). These enzymes show activities similar to *Aspergillus fumigatus* orthologues (20). Dermatophytes were also found to secrete a carboxypeptidase of the MEROPS M14A subfamily that is homologous to the human pancreatic carboxypeptidase A (21).

In a protein medium at acidic pH, dermatophytes secrete an aspartic protease of the pepsin family (Pep1) as an endoprotease, as well as tripeptidyl peptidases of the sedolisin family, prolyl peptidases and carboxypepti-

dases of the S10 family, and exoproteases (14). Analysis of the repertoire of secreted exoproteases in dermatophytes suggests basic mechanisms of extracellular proteolysis similar to those known in *Aspergillus* species at acidic and neutral pH (22, 23).

The proteases secreted *in vitro* are considered as virulence factors. However, transcriptome analysis performed with RNA from guinea pigs infected by the dermatophyte *Trichophyton benhamiae* and proteomic analyses of proteins extracted from infected nail beds of patients with onychomycosis revealed that a particular subtilisin (Sub6), not detected *in vitro*, was the major protease during infection (24, 25). Additional secreted proteins were detected, including the closely related Sub7 and the dipeptidyl peptidase DppV. Surprisingly, most of the proteases secreted *in vitro* during keratin digestion were not detected during the establishment of an infection. The results from *in vivo* experiments are of interest because Sub6 and DppV in *T. rubrum* were previously identified and described as major dermatophyte allergens, Tri r 2 and Tri r 4, respectively (26).

Other secreted hydrolases, e.g., ceramidases and lipases, are also putatively involved in degradation of the skin barrier. Microarray analysis revealed upregulation of genes such as heat shock proteins and transporters during *Arthroderma benhamiae* infection in guinea pigs, but the precise role of these genes in pathogenicity remains unclear (24). Marked upregulation of genes encoding enzymes of the glyoxylate cycle (i.e., isocitrate lyase and malate synthase) focused attention because this cycle was found to be necessary for virulence in *Candida albicans* (27) and for persistence in macrophages in *Mycobacterium tuberculosis* (28). However, virulence of deletion mutants defective in malate synthase was not attenuated in guinea pigs (29). The effects of the absence of the glyoxylate pathway might be suppressed by other metabolic pathways during infection.

Clinical Manifestations Distant from Dermatophyte Infections: Asthma and Skin Dermatophytides

Dermatophyte infections can provoke secondary immune reactions such as severe asthma and eczematous skin reactions (dermatophytids) in susceptible individuals. Dermatophytids, like *Trichophyton*-related asthma, can be controlled with systemic antifungal therapy.

Dyshidrotic and vesicular eczema on the hands (palms and/or fingers) associated with tinea pedis and/or tinea unguium in adults (generally caused by *T. rubrum* and *Trichophyton interdigitale*) are common dermatophytids (30). Eczema on the chest, trunk, and back associated with scalp ringworm due to zoophilic and anthropophilic species is less frequent but has been reported (31–33). A set of diagnostic criteria was developed to identify a dermatophytid reaction (33, 34): (i) there is a proven dermatophytic infection in a body site other than the locus of the eczematous skin reaction; (ii) the eczema appeared after the dermatophyte infection. The fungus is not present in the site of the cutaneous eruption. From this site, culture assays remain sterile and direct mycological examination is negative; (iii) the dermatophytid symptoms only resolve after eradication of the primary focus of fungal infection; (iv) the patients are sensitized to dermatophyte antigens and show a positive skin test response to fungal extracts (termed "Trichophytin"). In contrast to knowledge on *Trichophyton* asthma (35–37), individual antigens involved in dermatophytids have not been identified. Proteins secreted *in vivo* by dermatophytes are the best candidates as antigens involved in the host immune response.

Comparative Genomics

Genomes of dermatophytes are somewhat smaller than usual in filamentous ascomycetes, ranging from 22.5 Mb for *T. rubrum* to 24.1 Mb for *Trichophyton equinum* (38). Important functions concern secondary metabolism, proteases, and LysM binding domains, i.e., motifs that bind to various types of peptidoglycan and chitin. In contrast, genes involved in sugar metabolism and plant cell wall degradation are relatively depleted. There are only a few low-GC (31 to 36%) transposable elements, representing 1.3 to 7.2% of the genome (38), with low GC. Genomes of dermatophytes are colinear among each other, with only a few inversions. The core set comprises 6,168 orthologous groups of which 308 are unique to the dermatophytes. Species-specific genes have little functional information, and, hence, different clinical predilections between species cannot be explained with genome data alone. Three orthologous groups of the glycosyltransferase family (GT54) may be involved in immunomodulation of mannan upon infection. Burmester et al. (15) analyzed the global secretome during keratin degradation by growth with human keratinocytes. Dermatophytes produce a broad repertoire of genes encoding hydrolytic enzymes, lipases for cutaneous lipid degradation, and an exceptionally large number of proteases. Proteases do not differ significantly between species, indicating that proteolytic abilities are ancestral. Dermatophytes have an unusually high degree of nonreducing

polyketide synthases. Possibly this is linked to the often striking colony colors due to extracellular metabolites of the polyketide synthase pathway, such as the mycotoxin xanthomegnin, the red color of *T. rubrum*, which has been detected in clinical samples of epidermal material infected by *T. rubrum*, in contrast to noninfected controls.

Sexuality

Many geophilic and zoophilic species produce sexual phases on suitable media after confrontation of strains of opposite mating types, and, in such species, mating type distribution is balanced. Li et al. (39) matched molecular structure of the mating type locus idiomorphs (alpha and high-mobility group [HMG] domains) with *in vitro* mating results. Symoens et al. (40) showed balanced frequencies of + and − mating types in strains of the zoophilic species *T. benhamiae*. Anzawa et al. (41) showed mating of a highly competent *Arthroderma simii* tester strain producing a fertile F1 generation with a strain of *T. rubrum*, although, with a hybridization depression, only 1 of 35 ascospores was a real recombinant of the two species. On the basis of mating alone, some authors maintain rather wide species concepts uniting entities that are clinically and phenotypically different from each other (42). Another approach is a hypothesis of an atavistic ability to undergo genetic exchange via sexual reproduction/hybridization in response, e.g., the stressful conditions of a newly inhabited environment (13).

Over the phylogenetic tree, a gradual loss of sexual vigor can be observed. Kano et al. (43) confirmed that in the zoophilic species *Trichophyton verrucosum* idiomorphs of the mating type locus (alpha domain and HMG domain genes) exhibited only a single molecular mating type. Gräser et al. (Kosanke S, Hamann L, Gräser Y, unpublished data) noticed that this is the prevalent situation in several other zoophilic and most anthropophilic species. This suggests that anthropophilic species do not have an environmental niche where sexuality could take place, being transmitted between hosts. It may be hypothesized that clonal offshoots of interbreeding species are ecologically specialized and may be in danger of extinction upon changes in their niche on the human host (44).

Host Defense Mechanisms

Zoophilic and geophilic species of dermatophytes (e.g., *T. benhamiae*, *Trichophyton erinacei*, *T. verrucosum*, and *Microsporum canis*) cause highly inflamed lesions in humans. A dermatophyte provokes a more intense inflammatory reaction on a host to which it is not adapted than to its natural host; but on the other hand such lesions more rapidly lead to spontaneous resolution. Both innate and adaptive immunity are involved in host defense mechanisms against dermatophytes.

Innate immunity

Innate immunity in superficial dermatophytosis implies the action of keratinocytes and neutrophils. A broad spectrum of cytokines is produced by keratinocytes upon exposure to a dermatophyte (45), including interleukin-8 (IL-8), a potent chemoattractant for neutrophils that can kill dermatophytes, and the proinflammatory tumor necrosis factor α (TNF-α) (46). Production is higher in zoophilic than in anthropophilic dermatophytes (45, 47), which is consistent with the clinical features by the respective dermatophyte groups. Keratinocytes also secrete a wide variety of antimicrobial peptides with antifungal properties. Human β-defensin (48), cathelicidin LL-37 (49), psoriasin (50), and disulfide-reduced psoriasin (51) were proven to be either fungistatic or fungicidal *in vitro* against *T. rubrum*. Disulfide-reduced psoriasin was isolated from psoriatic lesions and confers resistance to fungal infections (51).

Zoonotic dermatophyte infections tend to be more inflammatory than those caused by anthropophilic species (52). Rare cases of deep dermatophytosis have been described in HIV and immunosuppressed patients (53, 54), but also in immunocompetent people, mainly from North African families with consanguinity (55). These patients were found to bear homozygous mutations in the gene coding for a caspase recruitment domain containing protein (CARD9). A stop codon mutation (Q289*) was detected in 15 patients from seven Algerian and Tunisian families (55). Two missense mutations, R101C and R101L, were detected in two Moroccan siblings and a Brazilian patient, respectively (55, 56). The mutation Q289* was also detected in a patient of Egyptian origin with extensive skin and nail dermatophytosis (57). CARD9 is an adaptor protein in the signaling pathway downstream from lectin receptors, such as dectin 1 and dectin 2 involved in the recognition of pathogenic fungi, and plays an important role in the innate immune response against fungal pathogens. The gene is expressed mainly in myeloid cells and is involved in the stimulation of proinflammatory responses. CARD9-deficient cells show low levels of IL-6 production after stimulation with zymosan, an agonist of dectin 1 (55).

Adaptive immunity

Zoophilic and geophilic dermatophytes induce a delayed type hypersensitivity (DTH) cell-mediated response,

which usually results in recovery and subsequent protection against reinfection. The response is characterized by elevated levels of the key cytokines IL-12 and gamma interferon (IFN-γ), which trigger T-helper 1 (Th1) cells for the activation of macrophages as effector cells. The overexpression of transforming growth factor β, IL-1β, and IL-6 mRNA during infection of *T. benhamiae* in a mouse model also suggests a role of the Th17 pathway in the establishment of immunity with recruitment of polymorphonuclear neutrophils (58). In contrast, anthropophilic species (e.g., *T. rubrum*, *T. interdigitale*, and *Trichophyton tonsurans*) tend to be associated with less inflammatory, but more chronic and persistent infections. These infections are correlated with poor specific DTH, elevated specific IgE and IgG4, and IgE-mediated immediate hypersensitivity, with the production of Th2 cytokines by mononuclear leukocytes (37, 59). While a cell-mediated Th1 response to dermatophytes is effective in eradicating the infection, Th2-mediated immediate hypersensitivity responses are not protective.

MALASSEZIA

Biodiversity

Malassezia species are among the most prominent microorganisms growing on human skin, where they form a significant part of the skin microbiome (60) thus corroborating older culture-based studies (61 and references therein). Because *Malassezia* spp. are extremely fastidious in culture, culture-based studies are potentially biased for the fast-growing and more robust species including *M. furfur*, *M. sympodialis*, and allies and against slower and fastidious species such as *M. restricta*, *M. globosa*, or *M. obtusa*. As such, in this review, we will focus on more recent DNA-based studies and point out specific differences where relevant.

It has long been known that all *Malassezia* species are lipophilic, and it has recently been shown that all species, including *M. pachydermatis*, are actually fully lipid dependent and lack a fatty acid synthase (9). They require long-chain fatty acid (e.g., C12 or longer) supplementation for growth (62). Within the Fungi, the genus *Malassezia* occupies a rather isolated phylogenetic position. Recent multigene phylogenetic studies placed the genus in its own class, *Malasseziomycetes* in the phylum *Basidiomycota*, subphylum *Ustilaginomycotina*, that otherwise mainly comprises plant pathogens (63). Taxonomically, the genus has been significantly enlarged during the last decades and currently contains 15 species (Fig. 3) (64–66). For a long time, only two species were recognized, namely the lipid-dependent and human-associated *M. furfur* and the then considered non-lipid-dependent and animal-associated *M. pachydermatis*. Major improvements in our understanding of the diversity and phylogenetic relationships of the *Malassezia* species resulted from molecular phylogenetic studies, especially those initiated by Guého et al. (67). The initial studies used the D1/D2 domains of the large subunit ribosomal DNA (LSU rDNA) and were later followed by the addition of the ITS (61, 68 and references therein). Improved phylogenetic insights based on these rDNA domains presently identify 14 species (66, 69). A recent phylogenomic study resulted in a fully resolved, well-supported phylogenetic tree in which four subclades were apparent (Fig. 4; 9). The most basal lineage is formed by *M. slooffiae* and *M. cuniculi* (clade C), with two larger terminal sister clades, namely clade A with *M. japonica*, *M. yamatoensis*, *M. obtusa*, and *M. furfur*, and clade B with two subclades, namely B1 with *M. pachydermatis*, *M. nana*, *M. caprae*, *M. dermatis*, and *M. sympodialis*, and B2 with the most common human skin commensal organisms, *M. restricta* and *M. globosa* (9). This core-eukaryotic gene tree, in general, agrees with a previous four-gene tree (70) and an amplified fragment length polymorphism (AFLP)-based tree (71).

Occurrence on Healthy Human Skin

Malassezia species have been considered commensal skin-inhabiting fungi that may be implicated in skin diseases (72). Their ubiquitous presence on humans has implied early maternal transmission, which is supported by a combination of culture and molecular studies (73). Recent advances in human mycobiome studies revealed that *Malassezia* species represent a commonly occurring group of fungi on and in the human body. When compared to bacteria, the abundance of fungi was generally lower, but *M. globosa* and *M. restricta* were more abundant near the ears and forehead (60). *Malassezia* species predominated on 11 body and arm sites of healthy individuals, namely the antecubital crease, back, external auditory canal, glabella, hypothenar palm, inguinal crease, manubrium, nare, occiput, retroauricular crease, and volar forearm. Eleven species were found to occur on various body sites (74), and a reanalysis of available metagenomics data from 12 individuals revealed that at least 12 *Malassezia* species can occur on skin. In terms of both frequency and abundance, in all individuals, *M. restricta* and *M. globosa* were the nondisputed numbers 1 and 2, with *M. sympodialis* ranking third (9).

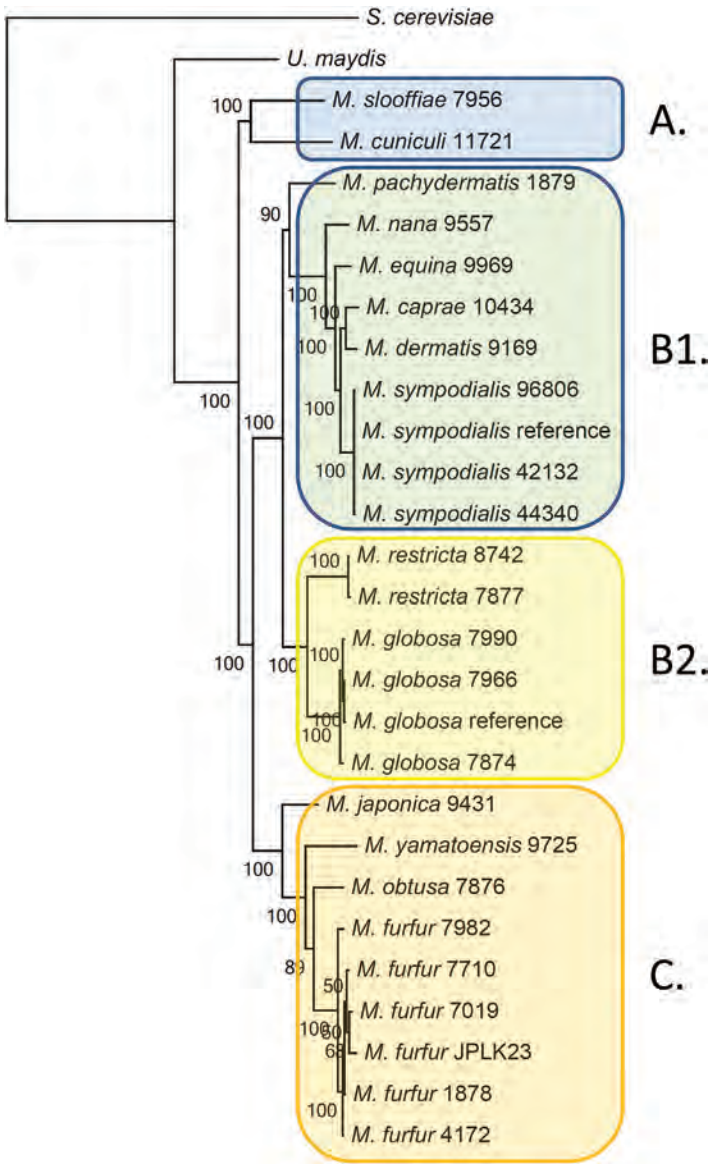


Figure 3 *Malassezia* species tree was constructed using concatenated sequences of 164 core eukaryotic genes that are present in all *Malassezia*, *U. maydis*, and *S. cerevisiae* genomes. Sequences were aligned using MUSCLE and the phylogeny constructed using a maximum likelihood (ML) approach by RAxML. RAxML was run using "–f a –m PROTGAMMAJTT" with 400 bootstraps.

Topological differences were observed. On the back, *M. globosa* was found to be most abundant on most individuals, with the exception of a single individual where *M. sympodialis* was dominant. The latter occurs in almost all cases as the second most abundant species on the back. In the antecubital or alar crease, *M. restricta* was most abundant in all subjects. Two of these species, and in many cases all three, were present in all individuals and also at most body sites sampled, but differences between individuals are observed. *M. slooffiae* occurred as a rather abundant species on one individual, whereas *M. obtusa*, *M. pachydermatis*, *M. caprae*, and *M. equina* were much less abundant. The occurrence of these latter species seems largely dependent on the individual. Interestingly, individuals showed the same spectrum of species present over a 3-month period (60). The factors that determine the presence of the rarer species are unknown. Many

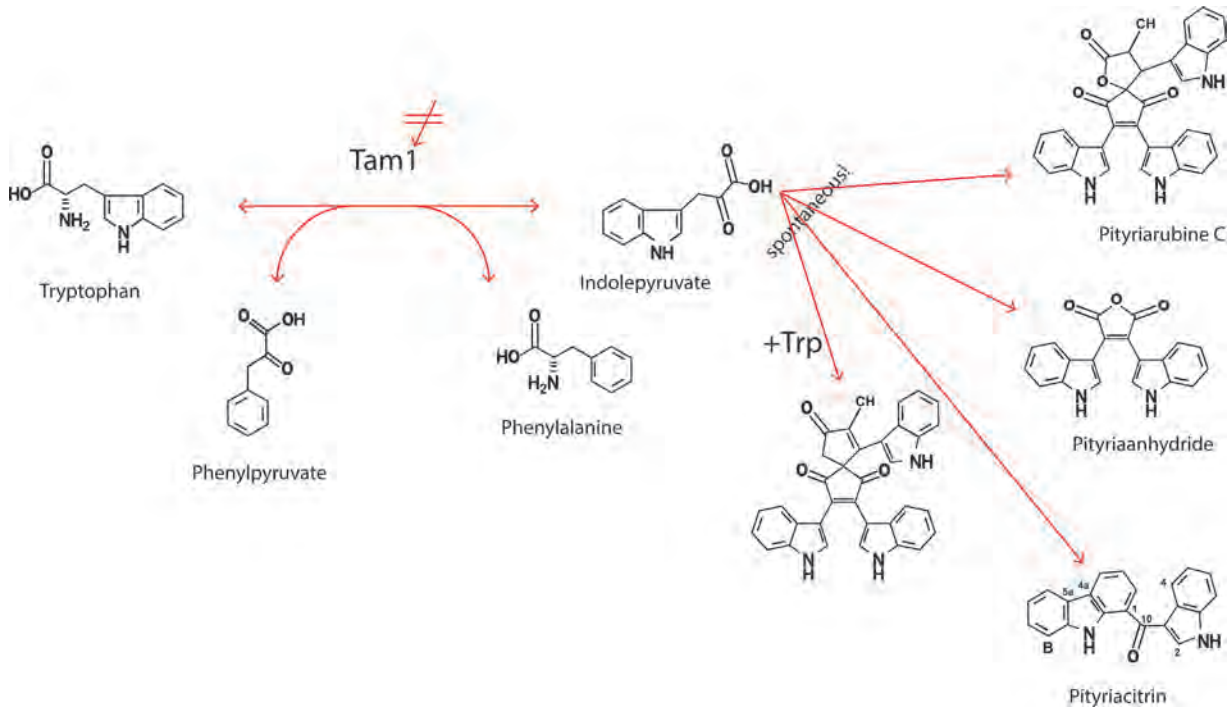


Figure 4 Synthesis of indole-derived pigments by one enzymatic step (TAM1) and possible intervention by TAM inhibitors.

more individuals from different ethnic and geographic origins, together with large-scale assessment of their skin conditions and other associated metadata, probably have to be investigated to understand any causal relationships. Furthermore, data as yet are primarily in Caucasians of Western descent and location, and more data will be required to understand the breadth of *Malassezia* diversity in other ethnicities and from people residing in different climates and geographies.

Occurrence in Human Skin Disorders

Malassezia spp. were found to be the most abundantly occurring fungi on both healthy and diseased skin with *M. restricta*, *M. globosa*, *M. sympodialis*, and *M. furfur* occurring on both and *M. slooffiae* only on nonlesional skin (75). The number of sequence reads of *M. restricta* and *M. globosa* differed significantly between both types of skin, with *M. restricta* being more dominant at lesional skin and *M. globosa* on nonlesional skin. No differences were observed in colonization of both skin types for *M. furfur*, *M. sympodialis*, or *M. slooffiae* (75). A French study that compared dandruff versus nondandruff scalp reported that the presence of dandruff correlated with a disequilibrium between *M. restricta* and *Staphylococcus epidermidis* or *Propionibacterium acnes* (63, 76). With the use of ITS and D1/D2 of LSU rDNA, it was found that *M. restricta* was the major fungus on both types of scalp with 97% of sequence reads from healthy scalp and 84% from scalp with dandruff (76). The difference in healthy versus dandruff scalp may also be due to intrinsic host factors separate from fungal metabolism, termed "individual susceptibility" (77).

M. globosa is a diverse species, the species most highly correlated with human disease and containing multiple genotypes (78). Four IGS1 genotypes have been described, and it has been suggested that one of those may be related to atopic dermatitis (79). The species is known from healthy skin, but also from lesions of PV and seborrheic dermatitis (SD) patients and animal skin (64, 80). Skin mycobiome studies revealed that *M. globosa* occurs on the back, occiput, and inguinal crease. The species may co-occur with *M. restricta* in SD/dandruff and atopic dermatitis patients (81). The abundance of *M. globosa* was significantly higher on SD than on healthy skin (75).

The second most important species on healthy and diseased skin, particularly of the head, including scalp, neck, face, and ears, is *M. restricta* (61). This species is known from the skin of healthy subjects, SD, dandruff, and atopic dermatitis patients (6, 81). It occurs abundantly on both SD and healthy skin (75), being isolated

from approximately 52% of SD patients compared to 64% in healthy humans (82).

M. sympodialis (83) is known from healthy human skin, in particular, the back and chest, but also from human PV, atopic dermatitis, skin, auditory tract, and various animals (64, 84). A recent Polish study using a culturing approach found that it was the most abundant *Malassezia* species on skin (85), but these results differ from many other studies that found *M. restricta* or *M. globosa* as the most common skin inhabitants. The discrepancy may have been caused by more ready isolation of *M. sympodialis* than of *M. globosa* and *M. restricta*.

Less commonly isolated species from human skin are *M. caprae*, previously known from healthy skin of goats and horses (86), and *M. dermatis*, known from lesions of atopic dermatitis and healthy skin (61). *M. furfur* has been reported from deep-seated infections, blood, urine, and vagina, and from septicemia in neonates that receive lipid supplementation via catheters (87, 88). Eight AFLP subtypes have been reported (68) that, however, did not fully coincide with subtypes based on sequences of the D1/D2 domains of the LSU rDNA. It has been suggested that isolates of AFLP genotype 4 may be more frequently involved in deep-seated infections, but the numbers that have been reported are low, hampering statistical analysis (71).

M. japonica is known from the skin of healthy Japanese women, but also from skin of atopic dermatitis patients (79). *M. obtusa* is known from the human groin, dermatitis skin, the nasal vestibule, but also from animals, such as goat, horse, and dog, and from canine otitis (89). *M. yamatoensis* is known from lesions of seborrheic and atopic dermatitis patients, but also from healthy human skin (64). It has also been suggested that several *Malassezia* species may be involved in skin carcinogenesis because of the production of aryl-hydrocarbon receptor ligands (90).

Occurrence Outside Human Skin

The mycobiome of the nasal vestibule of healthy humans and those suffering from allergic rhinitis is dominated by *Malassezia* species and comprises at least eight species with *M. restricta* as the most abundant and with *M. globosa* ranking as number 2. Further species observed were *M. cuniculi*, *M. dermatis*, *M. obtusa*, *M. pachydermatis*, *M. slooffiae*, and *M. sympodialis* (89). *Malassezia* species were found to be present with 100% colonization (91) and may also occur in the human gut, where five species were demonstrated using pyrosequencing of rDNA ITS (92). However, this approach is very sensitive and cannot discriminate between live resident flora and dead transient DNA. Using a PCR approach, *M. restricta*, *M. globosa*, and *M. pachydermatis* were found in stool of various individuals from different geographic locations, i.e., Amazonia, Polynesia, India, and Senegal (93). Another condition that has attracted interest is the prevalence of fungi in cystic fibrosis patients. *Malassezia* species, including *M. globosa* and *M. restricta*, were present in all sputum samples investigated, but with 10- to 50-fold lower levels than the more abundantly present *Candida* species (94). Finally, *Malassezia* species have been demonstrated to occur in the oral cavity where they have been suggested to be part of the basal oral mycobiome (95, 96).

A study using pyrosequencing of part of the small subunit ribosomal DNA (SSU rDNA) of sputum from asthma and nonatopic humans suggested that *M. pachydermatis* occurred commonly in asthmatic patients but was absent from healthy humans. A similar trend was seen for *M. furfur*, but the number of reads was much lower than that for *M. pachydermatis* (97). *M. pachydermatis* was also found in the nasal vestibules of healthy humans as well as those with allergic rhinitis (89).

Importantly, *M. pachydermatis* may cause catheter-related septicemia in neonates who receive lipid supplementation (98), and it also may cause sepsis in immune-suppressed adults. Domestic pet animals, especially dogs, have been identified as a source for transmission to neonates (99).

Occurrence Outside Humans

Many animals, such as cats, dogs, but also birds, horses, goats, cows, and pigs, may be infected by *Malassezia* species (65). *Malassezia* sequences have also been demonstrated from diverse terrestrial and marine ecosystems (100). Diverse *M. pachydermatis* isolates populate animal skin, including seven D1/D2 LSU rDNA genotypes that are known to exhibit some host specificity (101). Sequevar *a* originated from humans, sequevar *b* occurred in various animals, sequevar *c* came from rhinoceros, and cats and dogs were colonized by two and three genotypes, respectively.

M. equina was reported from healthy skin of a horse (anus) and cow in Spain (86), and *M. nana* from the otitis externa of cats, but also from cows in Brazil with or without otitis externa (102). *M. pachydermatis* is especially known from the external ear canal of many warm-blooded animals, especially dogs and cats, but also from bears, fox, rhinoceros, California sea lion, and many more (103 and references therein).

M. globosa is known from animal skin as well as from ears of healthy and diseased bovines and, for

the latter, a link with nematodes has been suggested (104). DNA of the species was detected in *Malenchus* nematodes in a European forest soil (105), and also in Antarctic soils where a connection with nematodes has been further postulated (106).

Biochemistry

Of the 15 currently recognized *Malassezia* species, *M. globosa* is thought to play a key role in the pathogenesis of PV (80). However, PV characteristics such as pigmentation alterations, fluorescence of PV lesions, or minimal signs of inflammation despite high fungal load (107) cannot be explained just by occurrence of *M. globosa*, which is also the predominant species on healthy skin. Instead, it was shown that, with tryptophan as a nitrogen source, *M. furfur* produces a number of indole pigments and alkaloids, which might be related to the clinical appearance of PV (Table 1) (108). Despite its complex biochemistry, the pathway does not represent secondary metabolism, as is the case with most of the mycotoxins. In the related and completely sequenced organism *Ustilago maydis*, it was shown that tryptophan and keto acids are converted to indole-3-pyruvic acid (IPyA) and the corresponding amino acids involving a tryptophan aminotransferase (Tam1; UM01804, XM_752858) as the initial and only enzymatic step. The indole pigments and alkaloids are then formed spontaneously from IPyA (109; Fig. 1). Tam1 of *M. furfur* (MfTam1) was shown to be differentially expressed under pigment-producing and nonproducing conditions (110, 111). The *mftam1* gene (1,431 bp) was cloned by a reversed PCR strategy (112). MfTam1 (molecular weight [MW] 52,500; JX 453496, EC 2.6.1.27) is specific for transamination of L-tryptophan with K_m values for the substrate and α-ketoglutarate in the low millimolar range (~7 mM). Its activity is highly dependent on pyridoxal phosphate (PLP) and compounds interfering with PLP, such as cycloserine (CS) and aminooxyacetate (AOA), inhibit the MfTam1 reaction. Mayser and Rieche (113) showed that a 200 mM CS solution topically applied for 5 days on PV lesions reversed the hyperpigmentation, and found with AOA tests the same effect as for CS on PV. However, AOA was more potent than CS (P. Mayser, unpublished data). Interestingly, comparison with the genome of *M. globosa* showed that the gene is also present in this species (MGL_2601, XM_001730167) (111). However, MgTam (MW ~35,500) shows a weaker activity *in vitro* than MfTam (Mayser, unpublished data). For CS and AOA inhibition of MgTam1 is expected, because the sequence is similar to the one of *M. furfur* and the inhibitors target PLP-dependent enzymes.

PV is very common in tropical climates and is associated with sweating and hyperhidrosis (114). The tryptophan concentration in sweat, however, is very low (~70 μM). It was shown that glycine metabolism of *M. furfur* results in *Malassezia* growth (115). However, metabolism of tryptophan is responsible for pigment production. When glycine and tryptophan are present, the fungi first grow and then, after a growth plateau is reached, start producing pigments. These data indicate that *M. furfur* preferentially metabolizes glycine. When the glycine concentration decreases, the metabolism switches to deamination of tryptophan with pigment formation as a by-product. In light of the fact that glycine is highly abundant in sweat (~2 to 4 mM) (116), it was hypothesized that severe sweating changes the local amino acid concentrations, resulting in an enrichment of the poorly water-soluble tryptophan on the skin. Thus, *M. furfur* (and probably *M. globosa*) might convert Trp to IP in a situation that lacks other nitrogen sources. The subsequently spontaneously generated indole pigments could be responsible for the clinical course of this common skin disease. TAM 1 inhibitors might represent a new approach in the therapy of this widespread disease.

Table 1 Selected Trp-derived indole compounds and their potential relationship to clinical phenomena in PV

Isolated compound	Pharmacological property	Phenomenon in PV
Pityriacitrin	UV protection	Lack of sunburn in depigmented areas of PV alba
Fluorescent metabolites (e.g., pityrialactone)	Fluorescence	Fluorescence of lesions in PV
Pityriarubins	Inhibition of oxidative burst in granulocytes	Lack of inflammation in lesions of PV
Malassezin	Induction of apoptosis in human melanocytes	Depigmentation in PV alba
AHR[a]-agonists (e.g., malassezin, pityriazepin, indolo[3,2-b]carbazole)	Activation of cytochrome, activation of AHR	Immunomodulation, effects on cell differentiation and homeostasis, depigmentation? desquamation?
Diversity of pigmented compounds	Pigmentation	Brick-red to brownish pigmentation of lesions in PV

[a]AHR, aryl hydrocarbon receptor.

Malassezia biology is also implicated in the common skin disorders dandruff and SD (9). These disorders are thought to be downstream of *Malassezia* lipid metabolism. In brief, *Malassezia* lacks the enzymes necessary to generate required fatty acids (no fatty acid synthase) or to assimilate unsaturated fatty acids (lacking a Δ9-desaturase). These deficiencies are compensated by the secretion of a number of hydrolytic enzymes that break down human sebaceous lipids. The *Malassezia* cells then assimilate the saturated fatty acids for survival (117), and leave behind irritating fatty acids that have been shown to induce dandruff-like desquamation in susceptible individuals (77).

Comparative Genomics

Twenty-four *Malassezia* isolates have been fully sequenced (Table 2), including all 14 currently accepted species and multiple isolates of the most common inhabitants of human skin (*M. globosa*, *M. restricta*, and *M. sympodialis*) and the most common human pathogenic species, *M. furfur* (9). The *Malassezia* genomes were compared with widely divergent fungal genomes, which identified features defining *Malassezia*. A large set of common fungal genes has been lost, corresponding to the compactness of the genome and adaptation to the skin environment. *Malassezia* genomics shows the genus has a propensity for gene turnover and highlights the importance of gathering nutrients from a sparse environment via secretion of large families of lipases, phospholipases, aspartyl proteases, and other peptidases. Recently, multiple genomes of *M. sympodialis* were generated via long read sequencing, and the genomes annotated via proteogenomics. This has significantly increased the reliability of the *M. sympodialis* genome annotation (118).

Several important facets of *Malassezia* warrant consideration. *Malassezia* produces a set of secreted hydrolases similar to *C. albicans*, a human skin pathogen, but is phylogenetically more closely related to the plant pathogen *U. maydis*. *Ustilago* produces a different set of secreted hydrolases, adapted for life on plants. The set of secreted enzymes utilized by each genus is likely

Table 2 *Malassezia* genome statistics[a]

Cluster	Strain	Genome (Mbp)	N50 (kbp)	Chromosome no. (PFGE)	Mitochondrial genome
Human skin	*M. globosa* 7966	8.9	724	9[b]	34689
	M. globosa 7990	8.9	415	9	38672
	M. globosa 7874	8.9	398	9	34808
	M. restricta 7877	7.2	403	10	38499
	M. restricta 8742	7.3	667	10	40172
	M. sympodialis 42132	7.5	54	8[a]	38623
	M. sympodialis 44340	7.5	60	8	38776
	M. sympodialis 96806	7.4	45	8	38538
	M. dermatis 9169	7.5	189	ND[c]	ND[c]
Animal skin	*M. caprae* 10434	7.6	110	ND[c]	ND[c]
	M. equina 9969	7.7	372	ND[c]	ND[c]
	M. nana 9557	7.6	492	ND[c]	ND[c]
	M. pachydermatis 1879	8.2	957	5[d]	28337
slooffiae-like	*M. slooffiae* 7956	8.3	16	9[b]	40575
	M. cuniculi 11721	7.5	522	ND[c]	ND[c]
furfur-like	*M. japonica* 9431	8.3	66	ND[c]	28009
	M. yamatoensis 9725	8.1	1,448	ND[c]	41233
	M. obtusa 7876	7.7	23	ND[c]	60147
	M. furfur 1878	13.5	15	10-11	48161
	M. furfur 4172	14	16	10-11	48279
	M. furfur 7019	13.4	16	8	49305
	M. furfur 7710	14.8	15	7	47901
	M. furfur JPLK13	8.5	1,649,308	7	ND[c]
	M. furfur JPLK23	7.6	15	7[b]	48933
	M. furfur 7982	7.7	21	7	47903

[a]Genomic data genebank accession: BioProject ID: PRJNA286710.
[b]Confirmed via PacBio sequencing.
[c]ND, neither pulsed-field gel electrophoresis (PFGE) nor PacBio sequence, only Illumina sequence available, mitochondrial genome not assembled.
[d]PFGE of pachydermatis 7925.

adaptation to the host niche and may be involved in pathogenicity (9, 72, 119). *Malassezia* species are all missing multiple enzymes required for robust lipid metabolism, including fatty acid synthase, Δ9-desaturase, and $\Delta^{2,3}$-enoyl-CoA isomerase. These gene losses likely explain their lipid dependence. Genuswide variation in these families also likely supports each species' niche specificity. *Malassezia* species have multiple gene duplications into several families. These genes have undergone lineage-specific duplications since divergence from *Ustilago* (9, 119). *Malassezia* species all encode proteins similar to the *M. sympodialis* allergens, supporting a role in skin and inhalational allergy. *Malassezia* genomes all have genes associated with mating, although mating has not been observed. They are likely among the first recorded fungi with a pseudobipolar mating phenotype (83, 120, 121).

Malassezia species have been shown to have gained and lost many specific gene families during their complex evolution. Of particular note is the likely gain of a single gene, conserved in all *Malassezia* but absent in any other sequenced basidiomycetes, suggesting a gene transfer at the very root of the *Malassezia*. The gene is likely functional, as it was found in *M. globosa* to be both transcribed and its transcription regulated. A second important potentially horizontally transferred gene family was located at the root of clades B1/B2 and was shown to be related to a catalase, which could adaptively protect *Malassezia* from a group of their own secreted proteins that generate hydrogen peroxide (9). Another example of *Malassezia* receiving genes via horizontal gene transfer is acquisition of a flavohemoglobin A from the *Corynebacterium* of *Actinobacteria*, potentially adding nitric oxide resistance into *Malassezia* (118).

Malassezia genomics also has revealed that they are likely to mate (9, 119). Fungal mating may be involved in pathogenesis, by increasing genetic diversity (122, 123). The mating loci suggest bipolar or pseudobipolar mating may be present in all *Malassezia* species. Studies using sequencing technology to build larger contiguous regions will be needed to define the mating systems in *Malassezia*, and further gene deletion or model systems to study any role in pathogenesis. Now that transformation and genetic engineering of *Malassezia* have been shown to be possible (124, 125), it is likely that the roles of these and other genes and pathways will be clarified.

Host Defense Mechanisms

As residents of the skin of all animals, and by definition then all human beings, *Malassezia* represents a unique opportunity to study the interaction of commensal microorganisms with the human immune system. As such, *Malassezia* has been shown to directly modulate cytokine expression by isolated human peripheral blood mononuclear cells (126). This report indicates that the *Malassezia* species most commonly found on human skin (*M. globosa*, *M. restricta*) downregulate expression of IL-6 or IL-1β, while the less common *M. sympodialis* does not. It was also indicated that the ability to modulate immune cells may be related to lipid metabolism, because extraction of *Malassezia* lipids via chloroform: methanol abrogated this effect. *Malassezia* lipids are further implicated in their relationship with the immune system in that the overall hydrophobicity of isolates correlates with their ability to stimulate secretion of proinflammatory cytokines from isolated human keratinocytes. An additional report further implicates a direct interaction with human monocyte-derived dendritic cells (127), where human NK cells activated monocyte-derived dendritic cells and induced an even stronger activation when *Malassezia* was present. Upregulation of CD83 and CD86 indicates that NK cells mature DCs and improve their costimulatory capacity.

M. sympodialis is able to directly elicit a human immune response, shown by the presence of human antibodies that cross-react with a group of *Malassezia*-secreted proteins termed "allergens" (128). In this series of reports, it was shown that individuals with specific forms of atopic dermatitis had serum IgG that could react with protein lysates from *M. sympodialis* and that subjects with head and neck atopic dermatitis had higher levels of anti-*Malassezia* IgG (129, 130). The allergens were identified, cloned, and sequenced, and later shown to be present and likely secreted in all *Malassezia* species (9). The *Malassezia* allergens are contained and secreted in nanovesicles, and the secretion and activity are modulated by skin pH (72).

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Fungal Biofilms: Inside Out

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INTRODUCTION

We focus this article on turning a biofilm inside out. The "inside" of the biofilm comprises the individual biofilm-related phenotypes, their environmental drivers and genetic determinants, and the coordination of gene functions through transcriptional regulators. Investigators have viewed the inside of the biofilm through diverse approaches, and this article will attempt to capture the essence of many. The ultimate goal is to connect the inside to the "outside," which we view as biofilm structure, development, pharmacological attributes, and medical impact.

Biofilms are surface-associated microbial communities, encased in self-produced extracellular material, that exhibit phenotypes distinct from those of planktonic (free-living) cells. Microbes are thought to grow predominantly as biofilms in nature (1). The surfaces with which biofilm cells are associated may be diverse and include solid abiotic materials, tissues and cells, and air-water interfaces. In fact, a colony growing on an agar plate is a biofilm.

Biofilm cells are quite different from the mid-logarithmic-phase planktonic cells that modern microbiologists were trained to study (see reference 2). Biofilm populations are invariably heterogeneous (Fig. 1A). Cells at the periphery of the biofilm are bathed in the external medium; cells at the base of the biofilm may have limited nutrients and oxygen and are surrounded by their neighbors' waste products. Cells at the periphery may be exposed to a rapidly fluctuating environment; cells at the base are buffered from many abrupt changes. Thus, a mature biofilm may include cells exposed to a range of nutrients, and the biofilm cell population may be growing at a range of rates.

Our focus on biofilms stems from their central role in infection biology (3). Biofilms are medically relevant in two major contexts: device-associated infection and *in vivo* growth. Device-associated infection is the phenomenon of the presence of an implanted medical device being a significant risk factor for bloodstream or deep-tissue infection. The specific risk factor and the likely types of infecting organisms vary with the kind of device and its location. The connection to biofilm formation was first elucidated by Costerton and colleagues (3), who found biofilms of infecting organisms on the devices that were removed from infected patients. In the vast majority of cases, the devices are sterile when implanted and later become colonized by microbes that enter the bloodstream. The biofilm on a device serves as a reservoir that continually seeds the infection. Unfortunately, as detailed later in this article, biofilm cells are generally recalcitrant to antimicrobials; thus, device removal may be the only therapeutic option. Because the usage of implanted devices continues to increase worldwide, the problem of device-associated infection will only grow in the future.

The second connection between biofilms and infection has to do with the nature of growth *in vivo*. Some infections are obviously surface-associated growth. Examples include mucosal infections such as thrush and vaginitis. Invasion of the surface may follow initial infection, and the surface itself may change over the course of infection due to inflammation and tissue damage. Other infections are not associated with host surfaces, but the infecting organisms grow as an aggregate encased in extracellular matrix (ECM) material. The aggregates may be considered self-contained biofilms, analogous to the flocs that form at the end of industrial fermentations (4).

We now look inside fungal biofilms at the major biofilm-relevant phenotypes and, where known, the molecular mechanisms that govern those properties. We focus primarily on *Candida albicans*, the biofilms of which have been studied extensively, as well as on *Aspergillus fumigatus*.

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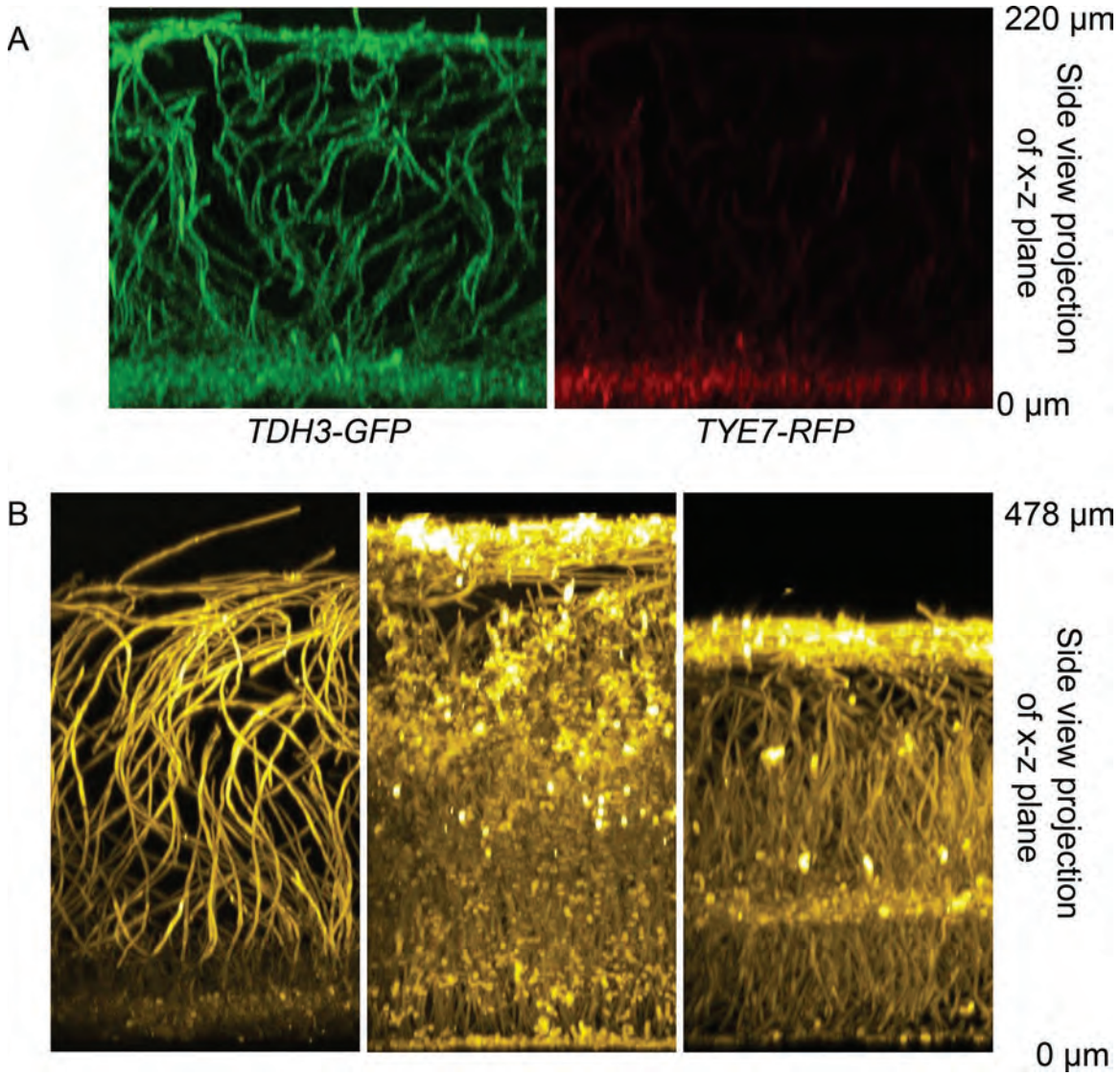


Figure 1 Confocal imaging of *C. albicans* biofilms. (**A**) Images show the heterogeneity of biofilms at the level of gene expression using a GFP construct fused to the *TDH3* promoter that is constitutively expressed throughout the biofilm and an RFP construct fused to the *TYE7* promoter showing greater expression at the basal layer of the biofilm, respectively. (**B**) Images show the phenotypic diversity of biofilms based on the growth medium in RPMI, YPD, and RPMI plus 10% serum, respectively. Biofilms were fixed and imaged using a Zeiss DuoScan confocal microscope. Side view projections were generated by reslicing and then z-projecting the stack using ImageJ software from the National Institutes of Health.

ADHERENCE

A necessary step in biofilm formation is attachment to a biotic or abiotic surface. For *C. albicans* biofilms, the attachment step is achieved through adhesive proteins expressed on the cell surface that are usually in the category of glycosyl phosphatidyl inositol (GPI)-anchored proteins. This kind of cell wall protein makes up a large portion of the total cell wall protein complement in *C. albicans*. Indeed, 115 putative GPI-anchored proteins have been identified, though more than half of these have no assigned function (5). The general structure of GPI-anchored proteins includes an N-terminal signal sequence, which traffics the protein to the endoplasmic reticulum, and a C-terminal hydrophobic domain site that allows the attachment of a preformed GPI anchor (6). A third structural part of GPI-anchored

proteins is the central region, which varies among proteins. Often, the central region contains repeats of conserved, functional units.

The Als (agglutinin-like sequence) family of adhesins is the most well-known category of GPI-anchored proteins in *C. albicans*. We use Als proteins to illustrate many general principles of fungal adhesins. Mutants defective in expression of *ALS1* and *ALS3* have decreased adherence to host and abiotic substrates (7–9). Due to their cell surface expression and role in virulence, recombinant Als1 and Als3 have been investigated for use as vaccines, with considerable success in mice (10). Als proteins contain the canonical GPI-anchor components along with an immunoglobulin-like region near the N-terminal domain, a conserved threonine-rich region, tandem repeats of a threonine-rich sequence, and a serine- and threonine-rich stalk that is often highly glycosylated near the C terminus (Fig. 2). Each of these domains has been studied in detail and has been shown to be functionally important (11).

The Als immunoglobulin-like region has two immunoglobulin-like domains and a peptide-binding cavity. This domain is responsible for binding to a broad range of host substrates such as E-cadherins, ECM proteins, or fucose-containing glycans (11–13). Although ligand binding by Als proteins in solution is relatively weak, the loss of a highly expressed Als protein has a very dramatic effect on adherence. This conundrum spurred further structural and biochemical analysis of the N-terminal regions of several Als proteins. Three Als regions are critical for substrate binding: the peptide-binding cavity, the threonine-rich region, and the tandem repeat region (Fig. 2). The peptide-binding cavity interacts directly with ligands (14, 15), and the other regions mediate Als protein aggregation, as discussed below.

Following the N terminus, there is a threonine-rich region (T). One function of this region is to promote secretion, though the mechanistic basis for this role is not understood (13). The second function is related to a highly conserved amyloid-forming domain within this region that mediates Als protein association, yielding polyvalent Als protein aggregates. This domain has been explored in detail by the Lipke group (16). Using atomic force microscopy and amyloid binding dyes such as thioflavin T, amyloid formation was observed with surface-expressed Als5. Following the discovery of amyloid formation in the Als proteins, Chan and Lipke made the connection that the adherence of fungal cells to surfaces is mediated through a process called "catch-bonding" (17). The process of catch-bonding seems to be broadly utilized by other organisms to mediate adhesion, because it has been described to occur with other systems such as mammalian selectins and bacterial adhesins (18). Chan and Lipke showed that the domains are activated through sheer force. The force causes nanodomains to form, comprising Als protein aggregates. This mechanism accounts for the strong adherence exhibited by *C. albicans* when it adheres and forms a biofilm compared to the relatively weak binding of adhesins to specific ligands in solution (17, 19).

The threonine-rich amyloid-forming region is followed by threonine-rich tandem repeats (TR). These repeats have been shown to be heavily glycosylated (13) and are predicted to form antiparallel β-sheets (20). Expression of Als5 derivatives that differed in the numbers of TR repeats in the heterologous host *Saccharomyces cerevisiae* has shown that they mediate cell-cell aggregation (13). This cell-cell aggregation function was independent of the immunoglobulin-like domain (13). Several modeling approaches argue that aggregation of the TR is mediated primarily by hydrophobic interactions (20). In addition, the TR region increases the affinity of the Als5 immunoglobulin-like region for at least one substrate, fibronectin (13). Spectroscopic analysis argues that this TR function is mediated by an effect on the folding of the immunoglobulin-like domain (13). It is remarkable that a region of such simple

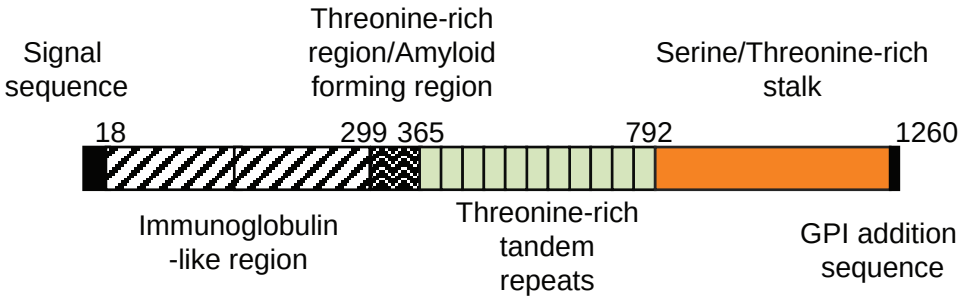


Figure 2 Als1 structure. Domains of Als proteins that are discussed in the text are depicted with amino acid coordinates of Als1 indicated.

sequence composition can have two distinct roles in Als5 activity. It would be interesting to know whether the two TR functions are separated genetically and if they are both critical for biofilm formation.

There is a serine- and threonine-rich region adjacent to the TR region. Mutations that reduce the length of this region prevent surface exposure of *S. cerevisiae*-expressed Als1 (21). These results are consistent with detailed work on the *Candida glabrata* adhesin Epa1 (22), which revealed that lack of the corresponding region caused the adhesin to be embedded within the cell wall rather than surface-exposed (22). The serine- and threonine-rich regions of several other *C. albicans* adhesins, Eap1 and members of the Hyr/Iff family, are also required for surface exposure (23, 24). Thus, the function of this region, to enable surface exposure of an N-terminal ligand-binding domain, seems to be similar among many adhesin-like proteins. This region is sometimes called the stalk, a name that seems to reflect its function quite well.

At the very C terminus of Als proteins (and many other cell wall proteins) is a short hydrophobic peptide that serves as a GPI-anchor addition signal. The GPI anchor, which is added in the endoplasmic reticulum, possesses a lipid tail that serves as a membrane tether. Upon trafficking to the cell surface, some GPI-anchored proteins remain tethered to the outer face of the plasma membrane, whereas others are transferred to become covalently linked to cell wall β-1,6 glucan (25). In *C. albicans* (26), as in *S. cerevisiae* (27), amino acid residues neighboring the GPI-anchor addition site determine whether the protein becomes localized primarily to the plasma membrane or cell wall. Beyond this generic function for the GPI-anchor addition signal, Ahmad et al. have provided evidence that this region influences the affinity of Als5 for its ligands (28). However, the study used recombinant proteins, and it will be exciting if this function can be demonstrated in live fungal cells. In general, the most broadly documented function for the GPI-anchor addition signal is to mediate cell wall attachment of adhesins and other mannoproteins (25).

How can you determine if a protein functions as an adhesin? Various approaches have been used to answer this question. One way to show adhesive function is to express the protein in a nonadherent organism. Fanning and colleagues utilized a *bcr1/* mutant strain of *C. albicans* that is defective in expression of most known adhesins, including Als1 and Hwp1 (29). Therefore, this strain does not adhere to surfaces under a variety of conditions. By overexpressing various genes of interest, Fanning et al. showed that several known adhesins (Als1, Als4, Als2) restored adherence in a *bcr1/* mutant.

The model yeast *S. cerevisiae*, like the *C. albicans bcr1/* mutant, does not adhere well to many surfaces. Therefore, it has been widely used as a heterologous expression system for assessing adhesive protein function. Als1, Als3, Eap1, Hwp1, and Rbt1 have each been heterologously expressed in *S. cerevisiae* and shown to be functional adhesins (30–32). The *S. cerevisiae* cells expressing these proteins attached and formed biofilms on abiotic and cellular substrates, albeit to varying degrees (30–32). Using *S. cerevisiae* to express *C. albicans* proteins is an effective way to reduce the redundancy of adhesins and other adherence mechanisms that are utilized by *C. albicans* cells. However, it is important to note that *C. albicans* translates the CUG codon as serine, while most other organisms including *S. cerevisiae* translate CUG as leucine (33). This coding difference may cause unintended structural consequences if the protein is not codon-optimized.

Biofilm integrity probably depends on a balance of cell-substrate adherence and cell-cell adherence (sometimes called cohesion [34]). The importance of this balance was emphasized in the studies by Cabral et al. of a panel of *C. albicans* strains that overexpressed 531 different individual genes, including many cell wall protein genes (35). They made the perplexing observation that overexpression of certain cell wall proteins affected a strain's ability to form biofilms differentially, based on whether the biofilm was a mono-culture or multistrain biofilm (35). For example, when cell wall genes were overexpressed in a pooled biofilm assay of 531 strains, the *PGA22*-overexpressing strain had increased abundance compared to other strains. However, when the strain was grown in a single-strain biofilm assay, the *PGA22*-overexpressing strain showed decreased biomass. The study found that overexpression of *PGA22* in *C. albicans* or a *PGA22*-derived hybrid gene in *S. cerevisiae* augmented cell-cell adhesion, and the resulting clumps of cells had increased sensitivity to shear force (35). Therefore, the flow conditions under which these biofilm assays were conducted could remove exposed cell aggregates in single-strain biofilms but could not dislodge the *PGA22*-overexpressing cells that were shielded by their 531 cousins in mixed-strain biofilms. This imaginative study illustrates the importance of a balance of adhesion to cells and substrates, as well as that cell-cell interactions within a biofilm can allow increased cell diversity.

The mold pathogen *A. fumigatus* also forms adhesive biofilms, though many of its properties are quite different from *C. albicans*. Biofilm formation in *A. fumigatus* occurs *in vitro* when grown under static aerial conditions or when grown attached to human

bronchial epithelial cells. The biofilm is characterized by a mat of hyphae encased in ECM and shows increased resistance to antifungal cells compared to planktonic cells (36, 37). For biofilms to form, these infections begin with inhaled conidia that adhere to bronchial epithelial cells or macrophages. *A. fumigatus* RodA was discovered as a hydrophobin—a surface protein that confers hydrophobicity—that is expressed on conidia (38). A *rodA* mutant was shown to have decreased adhesion but was dispensable for virulence in a mouse model of systemic infection (38). Similarly, CalA, a predicted adhesin, was shown to bind mouse lung cells and laminin (39). However, its role in virulence or biofilm formation remains to be determined. Current understanding of *A. fumigatus* biofilms suggests that an ECM component, discussed in the next section, may have a more significant role in biofilm formation than any cell wall proteins.

ECM MATERIAL

One of the hallmarks of all biofilms is the presence of ECM (3, 40). The ECM can have diverse functions, including surface adherence, cell-cell aggregation, and nutrient storage (41). Of special relevance to biofilm infections, ECM can present a barrier that buffers biofilm cells from antimicrobial agents as well as host defenses (42, 43).

ECM has been characterized from biofilms of several *Candida* species (summarized in reference 44). We focus here on two species, *C. albicans* and *A. fumigatus*, whose ECM has been studied in detail. We note that ECM abundance and composition can vary considerably with growth conditions (45), as does the biofilm structure itself (Fig. 1B), so some conclusions may differ between studies because of the use of distinct media.

Initial characterization of the *C. albicans* ECM through chemical analysis revealed the presence of glucose, hexosamine, protein, extracellular DNA (eDNA), and a few other components (46). This analysis was complemented through functional assays for biofilm integrity and adherence after enzymatic hydrolysis of ECM components (47). Overall biofilm integrity was found to depend on multiple ECM components, including β-1,3 glucan, chitin, protein, and eDNA.

Are there unique proteins in the ECM? A combination of electrophoretic separation and mass spectrometry identified abundant *C. albicans* biofilm matrix proteins and compared them to planktonic culture supernatant proteins (48). There was good correspondence between the samples, which included both conventionally secreted proteins, which have an N-terminal signal sequence, and unconventionally secreted proteins, which lack a signal sequence (49). No biofilm-specific ECM proteins were identified. Therefore, if any ECM proteins have unique functions in biofilm properties, these functions must arise in the context of ECM, perhaps as a result of high concentration or association with other ECM components.

The composition of *C. albicans* ECM has recently been defined comprehensively through a chemical and biochemical tour de force (50). The major components were proteins, carbohydrates, lipids, and eDNA. Most importantly, an abundant mannan-glucan complex called MGCx (sometimes pronounced "magic X") was identified. It comprised branched β-1,6 mannan complexed with linear β-1,6 glucan. Because this complex is present at low levels if at all in cell wall preparations, it cannot arise from inadvertent contamination of ECM with cell wall fragments. In fact, MGCx may be the first *C. albicans* biofilm-specific marker.

How might an ECM-specific marker like MGCx be assembled? A fascinating answer comes from a study in which candidate genes were used to make mutants defective in individual ECM carbohydrate components (51). One surprising result was that accumulation of three ECM carbohydrates—mannan, β-1,3 glucan, and β-1,6 glucan—is coordinated: mutations that reduce accumulation of one component generally reduce accumulation of all three. The most intriguing finding was that mixed biofilms comprising mutants blocked in production of different carbohydrate components displayed extracellular complementation, accumulating all components at levels as great or greater than the wild type. These findings indicate that ECM is assembled extracellularly (51). Perhaps the high local concentrations of substrates and enzymes for ECM assembly in the biofilm enable the reactions to proceed more rapidly than in the relatively dilute supernatant of a planktonic culture.

A second important finding from the comprehensive ECM analysis came from proteomic studies of biofilms formed *in vivo* in animal infection models (50). Specifically, although ECM from *in vitro* biofilms had 565 *C. albicans* proteins, ECM from *in vivo* biofilm models (venous catheter, urinary catheter, and denture) had only 16 *C. albicans* proteins. The vast majority of ECM proteins found *in vivo* were from the host. Therefore, host proteins may make unique functional contributions to the ECM from infecting biofilms. Interestingly, Nett et al. found a group of 14 host proteins in common among ECM samples from the three *in vivo* biofilm models (52), a very manageable number for functional assessments. The significance of the host protein contri-

bution is illustrated by the fact that an inhibitor of *C. albicans*-fibronectin interaction blocks biofilm formation *in vivo* in a catheter infection model (53). These exciting findings reinforce the importance of testing conclusions from *in vitro* studies in an *in vivo* biofilm model to validate their potential clinical significance.

A. fumigatus engages in biofilm growth during infection of the lung, where it clearly forms a cohesive multicellular aggregate enmeshed in ECM (54, 55). (*A. fumigatus* is also linked to some medical device-associated infections [56]). The *A. fumigatus* ECM comprises galactomannan, β-1,3 glucan, monosaccharides, polyols, melanin, and a small amount of protein (54). Importantly, *A. fumigatus* aggregates, called aspergillomas, resected from the lungs of patients contain both galactomannan and β-1,3 glucan, thus emphasizing the relevance to infection of these ECM components (55).

One of the major *A. fumigatus* ECM components is a polymer made of galactose and *N*-acetyl-galactosamine (galactosaminogalactan [GAG]). Comparison to *Aspergillus nidulans*, which makes a GAG that is low in *N*-acetyl-galactosamine, argues that *A. fumigatus* GAG has a major role in promoting biofilm formation (57). GAG is localized to *A. fumigatus* germ tubes and mycelia (58), and it mediates a multitude of virulence phenotypes. For example, it promotes adherence to abiotic surfaces through hydrophobic interactions and has been shown to interact with and bind to host cells such as pneuomocytes (58). GAG also interacts with the immune system and is necessary for resistance to killing by neutrophil extracellular traps (57). There is growing evidence that *C. albicans* ECM also functions in immune evasion and manipulation of the host response (59).

A. fumigatus ECM formed *in vitro* has some eDNA content (60, 61). Rajendran et al. found that chitinase activity accumulates in mature biofilms (60), and the association of chitinase with autolysis (62) suggests that autolysis leads to DNA release into the ECM. In support of this hypothesis, addition of a chitinase inhibitor reduced ECM eDNA content (60). DNAse treatment disrupts *A. fumigatus* biofilm integrity, thus indicating that eDNA plays a structural role in this species' biofilm (60).

DISPERSED CELLS

Dispersion is an important step in the developmental cycle of biofilm formation. The propagation of new biofilms necessitates the release and dispersal of new cells, because fungi are nonmotile. Uppuluri et al. measured the kinetics of dispersion of *C. albicans* cells using a flow biofilm model to mimic *in vivo* conditions where blood or other fluids create flow over implanted medical devices (63). They showed that even at early time points in biofilm formation, cells were being dispersed from the biofilm. Logically, the released cells were not composed of true hyphae, but the vast majority of cells were polarized yeast-form cells. Although dispersed cells were predominantly yeast, the cells showed unique phenotypic properties such as increased adhesion compared to planktonic yeast cells. The dispersed cells also displayed increased virulence in a mouse model of disseminated candidiasis. This suggests that the dispersed cells represent a distinct class of cell type in *C. albicans*.

Environmental signals can impact dispersion. Uppuluri showed that the amount of cell dispersion positively correlated with the amount of glucose in the media (63). Understanding the circumstances under which cells are most likely to be released from a biofilm and enter a patient's bloodstream could be important for clinical applications. Similarly, preventing dispersal of these highly virulent cells could be a way to combat systemic fungal infections caused by biofilms on implanted medical devices. In that context, promoter shut-off experiments have identified four regulators—Ume6, Pes1, Nrg1, and Hsp90—that govern cell dispersion from preformed biofilms (56, 63, 64). In addition, defects in a histone deacetylase complex encoded by *HOS2*, *SIF2*, *SNT1*, and *SET2* reduce the abundance of dispersed cells (65). These regulators and the genes they control may be useful pharmacological targets for limiting biofilm accumulation.

DRUG RESISTANCE AND PERSISTENCE

All biofilms are notorious for their resistance to antimicrobial drugs and antiseptics, and *C. albicans* biofilms are no exception. One of the most careful comparisons of resistance of *C. albicans* biofilm and planktonic cells, in the same assay format, comes from the pioneering studies of Hawser and Douglas (66). They observed that biofilm growth conferred 5- to 7-fold greater tolerance to amphotericin B, flucytosine, or several azoles. Time-course studies by Chandra et al. revealed that *C. albicans* biofilms develop resistance to amphotericin B, fluconazole, and other treatments rapidly—between 2 and 12 h after initiation of biofilm formation (67). How does biofilm resistance arise? Our current understanding is that several mechanisms contribute to this phenotype, which makes it robust and quite consistent among different *C. albicans* isolates and *Candida* species (66).

One factor that contributes to resistance is the upregulation of drug efflux pump genes *CDR1*, *CDR2*, and *MDR1* early in biofilm development (68, 69). Mutant analysis indicates that these genes have measurable impact on fluconazole resistance early in biofilm formation. As biofilms mature, though, other mechanisms make more prominent contributions to resistance.

A second factor is the presence of extracellular matrix material. One of the functional matrix constituents is DNA: it contributes to resistance to amphotericin B and caspofungin, as shown by the increased susceptibility of biofilm cells after DNAse treatment (70). Interestingly, the treatment did not affect fluconazole susceptibility, thus indicating that overall biofilm properties were not disrupted by the treatment. The mechanism by which matrix extracellular DNA confers resistance is not known. Soluble β-1,3 glucan is a second functional matrix constituent; it has a major role in biofilm azole resistance, as shown by Nett and colleagues through engineering altered expression of the β-1,3 glucan synthase gene *FKS1* (71). They observed that changes in *FKS1* expression were accompanied by corresponding changes in soluble β-1,3 glucan in the matrix. The phenotypic impact was dramatic: reductions in *FKS1* expression yielded biofilms that were extremely fluconazole-sensitive. The effect was biofilm-specific, in that the same strains were unaffected in fluconazole sensitivity during planktonic growth (71). Direct binding assays revealed that soluble β-1,3 glucan binds directly to fluconazole, thus indicating that drug sequestration is the mechanistic basis for biofilm fluconazole resistance (71). A follow-up study of candidate genes showed that three genes—*BGL2*, *PHR1*, and *XOG1*—also govern fluconazole susceptibility and matrix β-1,3 glucan production (72). These three genes specify extracellular glucan modification enzymes, making them inviting targets for antibiofilm therapeutics that might be combined with fluconazole.

Another biofilm feature that contributes to the resilience of biofilms against antifungals is the presence of persister cells. Persister cells are a distinct portion of dormant cells in a genetically identical population that can survive concurrent lethal rounds of antimicrobial treatments (73). *C. albicans* persister cells are unique in that they form only when grown in a biofilm (74); bacterial persister cells have been shown to form during both stationary phase and biofilm growth. This implies a distinct mechanism underlying persistence formation for fungal cells. When treated with amphotericin B or chlorhexidine, *C. albicans* cells grown to stationary or exponential phase are killed efficiently, but when the cells are grown as a biofilm they show a distinct biphasic curve of killing. Interestingly, *efg1/*, *cph1/*, and *mkc1/* mutants, which are defective in biofilm formation, still formed persister cells at consistent levels. This suggests that the mechanism of persistence formation is independent of formation of a normal biofilm; evidence points to the idea that attachment to a surface may be sufficient to induce persister formation (74). Additionally, the phenomenon of persister cells in *Candida* infections was shown to be clinically relevant through the screening of 22 patients that presented with long-term *Candida* infections. Perplexingly, the *C. glabrata* strains that were recovered showed low levels of persister formation. On the other hand, the *C. albicans* isolates formed high levels of persister cells (75).

One clue to the genetic basis of persister cells in *C. albicans* comes from Bink et al. (76), who showed that SOD (superoxide dismutase) mutants generate lower percentages of persister cells when treated with miconazole. SODs function by converting ROS (reactive oxygen species) to O_2–, thereby detoxifying the ROS. The reduction in persister cell formation was only true when using the antifungal miconazole, which had been shown to raise ROS levels in cells. However, these mutants did not completely eradicate the persister cells, so undoubtedly there is still research to be done to understand the genetic regulation of this phenomenon.

GENE EXPRESSION

There has been long-standing interest in the identification of genes that are induced during biofilm formation. Such genes were thought to be—and have proven to be—enriched for genes that contribute to biofilm formation, and they can be informative in terms of broader biological processes that are biofilm-relevant. As such, biofilm-associated genes can reveal the identities of major regulatory systems and environmental signals that act during biofilm formation. Below we call these genes "biofilm-associated genes" because they have been defined through many kinds of comparisons. Any one particular comparison may yield a distinctive set of genes that are upregulated in biofilm cells.

Is there a fundamental biofilm-associated gene expression pattern? There have been a few different approaches to this problem. García-Sánchez et al. profiled biofilm cells grown under diverse conditions in comparison to planktonic cells also grown under diverse conditions (77). They arrived at a set of 325 genes (out of only 2,002 represented on their microarrays) whose

differential expression was characteristic of biofilm cells from multiple comparisons; 214 genes were upregulated in biofilms. These 214 genes were enriched in transcription and translation genes as well as biosynthetic genes for amino acids, polyamines, nucleotides, and lipids. Upregulation of numerous translation-associated genes in biofilm cells was also observed in a comparison that looked for shared gene expression features among two wild-type strains, SC5314 and WO-1 (78). These findings argue that biofilm growth enables cellular biogenesis to occur over a prolonged period compared to planktonic growth in the same medium. A simple explanation is that a biofilm may capture excreted metabolites, thus facilitating their utilization when preferred metabolites are exhausted.

Many studies have reported that hyphal genes are upregulated in biofilms compared to planktonic cells (79–81). García-Sánchez et al. filtered those genes out by design through inclusion of a nonfilamentous mutant grown under biofilm and planktonic conditions (77); it was discovered that the mutant had the novel ability to form a biofilm on a glass surface. However, in the RNA-Seq biofilm-planktonic comparison of both SC5314 and WO-1, downregulation of hyphal genes was consistently observed in biofilm cells (78). The biofilm time-course analysis of Fox et al. (82) may explain these disparate results: at early times, hyphal genes were upregulated in biofilm cells, but at later times, they were downregulated, relative to planktonic hyphae. Perhaps sufficiently high concentrations of quorum sensing molecules such as farnesol accumulate in mature biofilms (83, 84), inhibiting hyphal growth and hyphal gene expression at late times.

How do gene expression features compare between biofilms grown *in vitro* and *in vivo*? Profiling of *C. albicans* catheter biofilms by Nett et al. (85) revealed some consistent responses. Specifically, amino acid biosynthetic genes and transcription/translation genes were upregulated in biofilm cells *in vivo*. In addition, numerous transporters were upregulated, suggesting that biofilm cells may be limited for nutrients. The study compared biofilm cells to planktonic hyphae, a likely basis for the failure to detect upregulation of hypha-associated genes in biofilm cells. Overall, then, the correlations among broad trends in gene expression indicate that *in vitro*-grown biofilms serve as a useful model for catheter biofilms that form *in vivo*.

Have biofilm-associated genes been validated functionally? In other words, is gene regulation a predictor of gene function? One approach is to use biofilm-associated gene properties to deduce biofilm-relevant regulatory pathways. The d'Enfert lab implemented this approach in their initial study of biofilm gene expression (77). Because many amino acid biosynthetic genes were upregulated in biofilm cells, they inferred that the general amino acid control regulator, Gcn4, may be critical for biofilm formation. Indeed, they observed that a *gcn4/* mutant produced a defective biofilm, one with reduced biomass compared to the wild-type strain (77). Another illustration of this kind of thinking comes from the observation that biofilm-associated gene expression resembles the response to hypoxia (86), an observation first reported for *Candida parapsilosis*. That observation suggests that a mutant defective in hypoxic regulation may be defective in biofilm formation. Indeed, Bonhomme et al. found that Tye7, a transcription factor required for the hypoxic response (86), is required for many features of *C. albicans* biofilms (87). A third example comes from the point raised above that hypha-associated genes have often been observed to be upregulated in biofilms (depending on the specific biofilm-planktonic comparison). That observation makes sense because hyphae are a prominent feature of *C. albicans* biofilms grown under almost any condition, and it suggests that mutants defective in expression of hypha-associated genes will be defective in biofilm formation. That prediction has turned out to be correct time and again (80, 88–90). Therefore, inferences from biofilm profiling data about biofilm-relevant regulatory pathways have proven successful in terms of functional validation.

The second approach to functional validation is to determine whether each specific biofilm-associated gene has a measurable function in biofilm formation. Here the results have been mixed, though there may be some useful lessons for the future. The initial study of this kind, from the d'Enfert group, examined deletion mutants of 38 biofilm-associated genes for defects in biofilm biomass or hyphal morphogenesis (87). The genes had been chosen based on the magnitude of their upregulation in biofilms after elimination of likely essential genes and members of gene families. Eight of the 38 mutants produced biofilms with moderately reduced biomass but had no planktonic growth defect; one mutant had severely reduced biofilm biomass and a severe planktonic growth defect. On its face, the results were slightly disappointing because the yield of biofilm-defective mutants was low and because the biofilm-defective mutants identified had mild defects. In a related approach, Desai et al. focused on genes that were upregulated in biofilms of two clinical *C. albicans* isolates (78). Of the 62 most highly upregulated genes, viable insertion mutants could be isolated for 25 genes. The mutants were screened for a panel of biofilm-

related phenotypes, including biofilm formation, drug tolerance, quorum-sensing signaling, and others. Most of these mutants, 20 of 25, had significant defects in at least one biofilm-related phenotype. The high yield of biofilm-relevant phenotypes in this study may have reflected both the gene selection criteria and the range and sensitivity of the phenotypic assays.

The validation approaches and results presented above apply to *in vitro*-grown biofilms. However, the specific *in vitro* conditions used can have dramatic effects on biofilm properties (34, 91), and some mutations cause a biofilm defect only under a subset of *in vitro* conditions (92). In fact, one theme that has emerged repeatedly in all aspects of infection biology is that *in vitro* conditions can be poor mimics of *in vivo* conditions. Hence, the key validation approach in our view is to determine whether a biofilm-associated gene can be shown to function in an animal model of biofilm infection. The most frequently used model is a rat venous catheter biofilm model (93). Several mutant strains have similar biofilm defects *in vitro* and *in vivo* (7, 56, 72, 78, 80, 94–96). However, there are examples in which the severity of a mutant defect is much greater *in vitro* than in a catheter model *in vivo*, and vice versa (78, 80, 97, 98). It would accelerate biofilm research considerably if there existed an *in vitro* model that could accurately predict *in vivo* outcomes.

BIOFILM GENE REGULATION

For three main reasons, it has proven useful to identify biofilm regulators. First, because a single transcription factor often controls many functionally related target genes, a single biofilm-defective transcription factor mutant can lead to discovery of many biofilm-relevant genes among the target genes that it regulates. One of the initial illustrations of this principle came from studies of the *S. cerevisiae* mating type locus, which specifies master regulators whose target genes confer individual cell type-specific properties (99). Second, there are many examples in which a transcription factor mutant has a more prominent phenotype than mutants defective in individual target genes of the transcription factor. A simple illustration comes again from studies of *S. cerevisiae*, this time studies of meiosis and spore formation: many mutations that caused a prominent sporulation defect affected meiotic regulators rather than the machinery that mediated specific meiotic events (100). This outcome could reasonably be considered a result of the first principle. Third, some biofilm regulators identified in *C. albicans* have orthologs in other *Candida* species that also govern biofilm formation (discussed below). Hence, a biofilm regulator can provide an entry point for definition of biofilm-relevant genes and biological processes in many organisms.

The transcription factors that control biofilm formation have been identified through several approaches. One approach is deductive logic based on expression profiling or other biofilm features, as discussed above (87–90). A second approach is to screen a set of transcription factor mutants for defects in biofilm formation or related phenotypes. For example, panels of both insertion and deletion mutants have been screened for failure to form biofilms (80, 82, 101). An elegant variation on this approach was to assay mutants at several time points in biofilm formation (82). Another related screen was for mutants that were defective in adherence to a silicone substrate (97). Overall, 51 transcription factor genes have been shown to affect biofilm-related properties in these large-scale screens as well as more focused studies (88, 102).

These studies have revealed that there is a major biofilm regulatory network, sometimes called the "core network" (102), comprising transcriptional regulators of hyphal morphogenesis and hyphal genes. These transcription factors include Flo8, Rfx2, Gal4 (82), Brg1, Bcr1, Rob1, Efg1, Ndt80, and Tec1 (80, 90, 101). They are functionally interconnected, in that each binds to at least one other network regulator's upstream region and regulates its expression (80, 82). Rfx2 and Gal4 are negative regulators of biofilm formation; the respective null mutations cause increased biofilm formation (82), and for Rfx2, an increase in expression of many hyphal genes (103). The other transcription factors in this network are positive regulators of biofilm formation; null mutations cause reduced biofilm formation and reduced expression of hyphal genes (80, 82, 90, 101). For many of the positive regulators in this network, current evidence indicates that adhesin genes *ALS1*, *ALS3*, and *HWP1* are critical downstream target genes, because overexpression of one of these genes can restore considerable biofilm formation (7, 9, 80). However, there are likely to be additional network target genes that contribute to biofilm formation, because phenotypic rescue by adhesin overexpression is only partial in some cases (80). There are 21 transcription factor genes whose *5′* regions are bound by one or more network regulators and that govern biofilm-relevant phenotypes (*AHR1*, *BPR1*, *CAS5*, *CRZ2*, *CZF1*, *FCR3*, *GCN4*, *GRF10*, *GZF3*, *MSS11*, *NRG1*, *RFG1*, *RIM101*, *TRY4*, *TRY5*, *TRY6*, *TYE7*, *UME6*, *ZAP1*, *ZCF31*, and *ZCF8*) (88, 102). Hence, this regulatory network controls adhesins and, potentially, many additional diverse biofilm properties.

Are all biofilm properties under the control of this core network? It is not clear as of yet. There are 21 transcriptional regulatory genes that control biofilm formation or biofilm-relevant properties, including *ACE2*, *ADA2*, *ARG81*, *DAL81*, *FGR27*, *LEU3*, *MET4*, *NOT3*, *RLM1*, *SNF5*, *SUC1*, *TAF14*, *TRY2*, *TRY3*, *UGA33*, *WAR1*, *ZCF28*, *ZCF34*, *ZCF39*, *ZFU2*, and *ZNC1* (102). These regulators govern such properties as the level of ECM (104) or the adherence of yeast-form cells (97). These features could conceivably be regulated independently of hyphal adhesins. Whether they are regulated through the core network is an interesting question for future studies.

Have we found all of the biofilm regulators? One could reasonably argue that 51 regulators are enough. We suspect that there are others, though, and that they are worth studying because of the *in vitro-in vivo* differences mentioned earlier in this article. Specifically, our lab has identified transcription factors that are required for biofilm formation *in vivo* but not *in vitro* (97). Thus, we hypothesize that the systematic screens used so far may have overlooked other transcription factors with these properties. Perhaps they could be identified through application of the elegant mutant pool screens used by Noble (105) to assay *in vivo* biofilm formation assays.

CONSERVED FEATURES

Are there conserved features among biofilms of different *Candida* species? There are certainly some conserved features, even though the biofilm architecture of each species has been shown to vary. Several researchers have investigated this question of conservation by comparing *C. albicans* and *C. parapsilosis* biofilms. Evidence suggests that the major regulators of biofilm formation are conserved by both species. This may come as a surprise, because *C. parapsilosis* does not form true hyphae. Ding et al. (106) showed that the transcription factor Bcr1 is a major regulator of biofilm formation in *C. parapsilosis*, comparable to *C. albicans*. Also, some downstream targets of Bcr1 are conserved between the two species. Specifically, Bcr1 was shown to regulate genes encoding a family of proteins called CFEM (Common in Fungal Extracellular Membrane) in both species. The CFEM proteins have been implicated in biofilm integrity (107, 108) in *C. albicans*, but Ding found no evidence that CFEM mutants in *C. parapsilosis* are defective in biofilm formation *in vitro* (106). Several CFEM proteins also function in heme iron acquisition (109), and their regulation by Bcr1 is consistent with the iron utilization defect of the *C. albicans bcr1/* mutant (106). However, the specific Bcr1-regulated genes that are required for *C. parapsilosis* biofilm formation remain to be identified.

Holland et al. were the first to make an extensive deletion collection in *C. parapsilosis*, which included homozygous deletions of 100 different genes, including many transcription factor genes (110). From this study, many of the phenotypes were shown to be conserved between *C. albicans* and *C. parapsilosis*. In the roles of biofilm formation, Efg1, Bcr1, and Ace2 have all been confirmed as major regulators in both *Candida* species. However, Holland found several other transcription factors (Cph2, Czf1, Gzf3, and Ume6) that played a role only in *C. parapsilosis*. Upon sequencing of the biofilm transcriptome from each species, Holland found that similarities between the two species clustered around genes involved with metabolism (110). Similarly, when biofilms of both species were grown under hypoxic conditions, there was good correlation between the two transcriptional profiles (86). For example, Upc2 is required in both species for regulation of ergosterol biosynthesis genes during hypoxia (111). It seems that the transcriptional landscape of biofilm formation in *C. albicans* and *C. parapsilosis* may be broadly conserved, but with some distinct divergences among regulators.

CONCLUSION

Inspection of the inside-out biofilm leads to a simple question: Will a biofilm turn out to be simply the sum of the parts? It is almost certainly not the sum of the parts that we know at this time. We still have limited understanding of the drivers and determinants of cell heterogeneity, as well as the flip side of heterogeneity, which is cell-cell cooperation. Biofilms, like tissues, present a considerable systems-level challenge. We often conceive of the collection of genes that govern biofilm formation as a network that acts within a single cell, much like the systems-level view of *S. cerevisiae*. However, consideration of cell heterogeneity makes a compelling case to us that many different networks are active among the diverse cells of a biofilm.

An additional challenge that underlies the sum-of-the-parts question is that our biofilm parts list is incomplete. One reason it is incomplete is because our *in vitro* biofilm models fail to recapitulate many features of *in vivo*-grown biofilms; hence, mutant phenotypes differ *in vitro* and *in vivo*. Each kind of biofilm—venous catheter, mucosal, etc.—is likely to reflect a distinct balance of biofilm phenotypes and regulatory inputs. Going forward, it seems imperative to validate experi-

mental results with *in vivo* models to see which biofilm determinants are most relevant to infection biology.

Despite those challenges, a brief reflection on the history of fungal biofilm research underscores our optimism and excitement for the future. It was only about 20 years ago that Julia Douglas' lab first viewed *C. albicans* biology through the lens of the biofilm paradigm. Investigators have since elucidated the genetic and biochemical determinants of the many biofilm features discussed in this chapter. We now have a solid foundation from which to build our understanding of a fungal biofilm from the inside out.

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Fungal Recognition and Host Defense Mechanisms

43

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CONCEPTUAL FRAMEWORK FOR IMMUNOLOGY

The immune system has over millennia evolved strategies to discriminate between what is "self" and what is foreign (nonself) as well as "normal self" and "injured or altered self," with the ultimate purpose being to defend and repair self. First, to fully appreciate the framework that governs most of our current understanding of how the immune system operates, it is imperative to be cognizant of some of the immunological models that have laid the foundation on which we stand.

Self-Nonself Discrimination Models

The 1960 Nobel Prize in Physiology or Medicine was awarded to F. MacFarlane Burnet and Peter Medawar for the discovery of immunological tolerance. Burnet suggested that each B lymphocyte, an immune cell responsible for the production of antibodies (or immunoglobulins), expresses numerous clones of cell surface immunoglobulin receptors specific for a foreign body (antiforeign body) and that when antibody receptor recognizes its cognate antigen, an immune response is initiated (1). This idea was solidified by Billingham, Brent, and Medawar's findings, which demonstrated that adult mice "tolerate" skin grafts if they are transplanted at birth; that is, the immune system can learn to tolerate the foreign antigens until defensive intolerance is acquired (2).

The self-nonself discrimination model became the *modus operandi* of the immune system until it was modified in 1969 by Bretscher and Cohn, who postulated that a quiescent B lymphocyte requires interaction with a "helper" cell (now known as helper T lymphocyte) specific for the same antigen to induce an activation signal, whereas inactivation ensues when antigen is recognized in the absence of the helper cell (3). In 1975, Lafferty and Cunningham introduced a requirement for a costimulation signal and proposed that recognition of antigen by the helper T cell (which provides *signal 1*) together with a "stimulator cell" (*signal 2*) (known now as antigen-presenting cell [APC]) induced activation of the immune system (4, 5). Over a decade later, Jenkins and Schwartz provided experimental evidence for the requirement of costimulation for activation of resting lymphocytes (6). However, the prerequisite of costimulation introduced a conundrum because this meant that immune activation hinges on APCs that capture and present both self and nonself antigens; therefore, immunity could not be solely targeted against the invading foreigners.

Noninfectious Self and Infectious Nonself Model and the Inception of Pattern Recognition Receptors

Charles Janeway provided an elegant solution to the costimulation conundrum mediated by APCs. In his 1989 article Janeway suggested that the immune system has evolved specifically to recognize and respond to infectious organisms and that this involves recognition not only of antigenic determinants but also of certain characteristics or patterns common in infectious organisms but absent from the host, and he coined the term "pattern recognition receptor" (PRR) (7). He also pointed out that these germline-encoded receptors would act early during an immune response and imagined that they also play a role in shaping the adaptive T and B cell responses. Another piece of the puzzle was added by Polly Matzinger, who in 1994 proposed

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that resting APCs are activated by stimuli associated with host-cell damage, which she termed "danger signals" (8). She posited that by definition, pathogens damage the host and that recognition of damage could be instrumental in validating initiation of an immune response. A summary of the history of the immune models discussed above is depicted in Fig. 1.

PRRs INVOLVED IN SENSING FUNGI

Several classes of PRRs have been discovered since their initial identification, providing experimental support for Janeway's theory. It is noteworthy that pioneering work emanating from the use of fungi and their antigenic determinants has contributed significantly to our understanding of the initiation of an immune response (for a detailed timeline, see reference 9). Here, we will discuss PRRs that have been demonstrated to sense the presence of fungi and activate antifungal host immune responses as well as the implications of defective receptor/signaling networks on susceptibility to fungal infections.

Due to the ubiquitous presence of fungi in the environment, humans are constantly exposed to them; in fact, it has been proposed that we have coevolved with commensals such as *Candida* spp. and *Malassezia* (10). Despite this, fungi are associated with various diseases ranging from asthma to mucocutaneous infections to severe life-threatening systemic infections in patients with a compromised immune system (e.g., organ tissue transplant patients and HIV-infected individuals).

Fungi are eukaryotes and thus are similar to mammalian cells; however, they possess a cell wall, which is absent in mammalian cells. The cell wall is thought to provide several advantages to fungi including protection against environmental stresses and structural rigidity for invading ecological niches (11). For many fungal species, the molecular details of the structural composition and organization of cell wall components have not been fully elucidated. However, for most of the medically relevant fungi, polysaccharides account for more than 90% of the wall content, with the central core comprising branched β-1,3 and β-1,6 glucan that

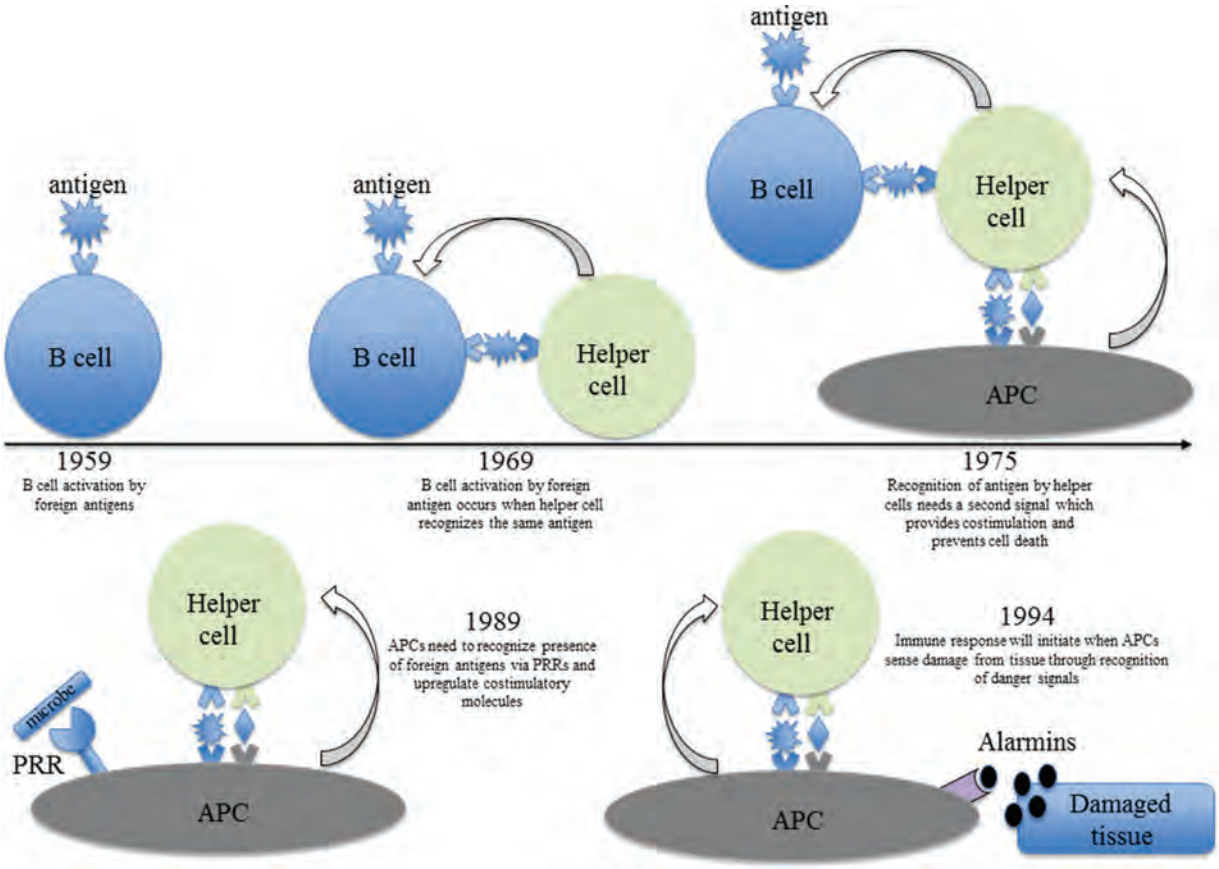


Figure 1 More than half a century of immunogical theories.

associate with chitin via a β-1,4 linkage (11). The core structure is decorated by unique extensions depending on the pathogen; for instance, *O*- or *N*-linked mannans and mannoproteins are found in *Candida* yeasts, while glucuronoxylomannan and galactoxylomannan coat *Cryptococcus* (12). Because of their exclusivity, the components of the fungal cell wall contribute a large reservoir of Janeway's "patterns," which are now commonly termed "pathogen-associated molecular patterns" (PAMPs). Chitin, mannans, and β-glucan are the three major PAMPs present in all fungi that cause human disease, with the possible exception of *Pneumocystis jirovecii* which lacks chitin (13, 14). Thus, the framework for induction of an immune response to most fungi follows similar sets of rules and involves recognition of PAMPs by several classes of germ-line-encoded PRRs expressed by innate immune cells, including C-type lectin receptors (CLRs), Toll-like receptors (TLRs), nucleotide-oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), discussed below.

TLRs and Fungal Sensing

By the mid-1980s, the nuclear factor-κB (NF-κB) was known to mediate upregulation of many genes involved in immunity and stress in mammalian cells (15). In *Drosophila melanogaster*, the NF-κB homologue, Dorsal, was shown by Nüsslein-Volhard and colleagues to drive expression of genes involved in development (16). Furthermore, activation of Toll by its ligand Spätzle was found to be the critical receptor involved in triggering the signaling cascade resulting in activation and nuclear translocation of Dorsal (17). Notably, work emanating from the Hoffman laboratory also demonstrated the expression and nuclear translocation of Dorsal upon microbial challenge (18). Importantly, this group demonstrated that the Spätzle-Toll-Dorsal signaling axis mediated expression of the antifungal peptide drosomycin, with Toll mutant flies succumbing to infection with *Aspergillus fumigatus* (19). Of interest to note is that *Drosophila* possesses only an innate immune system, while mammals also have an adaptive immune system. Medzhitov and Janeway subsequently demonstrated that a human cDNA clone of Toll homologue (now known as TLR4) also activated NF-κB and induced expression of costimulatory molecule B7.1 required for activating the adaptive immune responses (20). Seminal findings from Buetler's laboratory using mice with genetic mutations that abolished recognition of lipopolysaccharide, a major PAMP conserved in Gram-negative bacteria, established that TLR4 is the innate immune sensor of infection critical for host immunity (21).

Since the discovery of TLR4, over 12 mammalian TLRs have been described and their ligands and functions in immunity extensively studied (22). Similar to *Drosophila* Toll, the extracellular domain of TLRs contains leucine-rich repeat motifs and the intracellular domain structure that is homologous to the interleukin-1 (IL-1) receptor and pathogen resistance proteins found in plants—hence the name TIR (Toll/IL-1 receptor/resistance domain) (23, 24). TLRs transmit signals by interacting with several TIR-containing proteins such as myeloid differentiation primary response (MyD88), TIR domain-containing adaptor protein inducing interferon-β (IFN-β) (TRIF), and TRIF-related adaptor molecule (Fig. 2). With the exception of TLR3 and to some degree TLR4, all TLRs couple the MyD88 adaptor, which interacts with the IL-1 and IL-4 receptor-associated kinase (IRAK1/4) via its N-terminus death domain, resulting in downstream activation of several transcription factors including NF-κB, activation protein 1, interferon regulatory factor (IRF) 3, IRF5, IRF7, and mitogen-activated protein kinases that induce expression of inflammatory mediators (25).

Mice lacking MyD88 have been shown to be sensitive to infections with various pathogens, presumably, in addition to TLR unresponsiveness, defective immune activation due to a lack of IL-1R, IL-18R, and IL-33R signaling, which also utilize the TIR-MyD88 module (26–28). In experimental settings, MyD88-deficient mice displayed broad susceptibility to bacterial, viral, parasitic, and fungal pathogens, although only selected bacterial infections were observed in humans with deficiencies in MyD88 or IRAK4 (see reference 29 for a detailed review). Nonetheless, polymorphisms in TLR4 were found to be associated with pulmonary infections with *Aspergillus* and systemic candidiasis (30–32). Additionally, mutations in TLR3 resulting in reduced activation of NF-κB and decreased production of IFN-γ were associated with cutaneous candidiasis (33). Furthermore, defective TLR9 expression was implicated in allergic bronchopulmonary aspergillosis in humans, suggesting a possible accessory role of TLRs in antifungal mammalian immunity (32). Thus far, fungal PAMPs including α-1,4-glucan, glucuronoxylo/phospholipido-mannans, cytosolic nucleic acids, and *O*-linked/rhamnomannans have been reported to be recognized by TLRs (12) (Fig. 3).

Contribution of NLRs and RLRs

NLRs comprise a large family of cytoplasmic receptors made up of three characteristic domains (NH_2-terminal protein-protein interaction domain, a central NOD, and a C-terminal leucine-rich repeat) and can be grouped

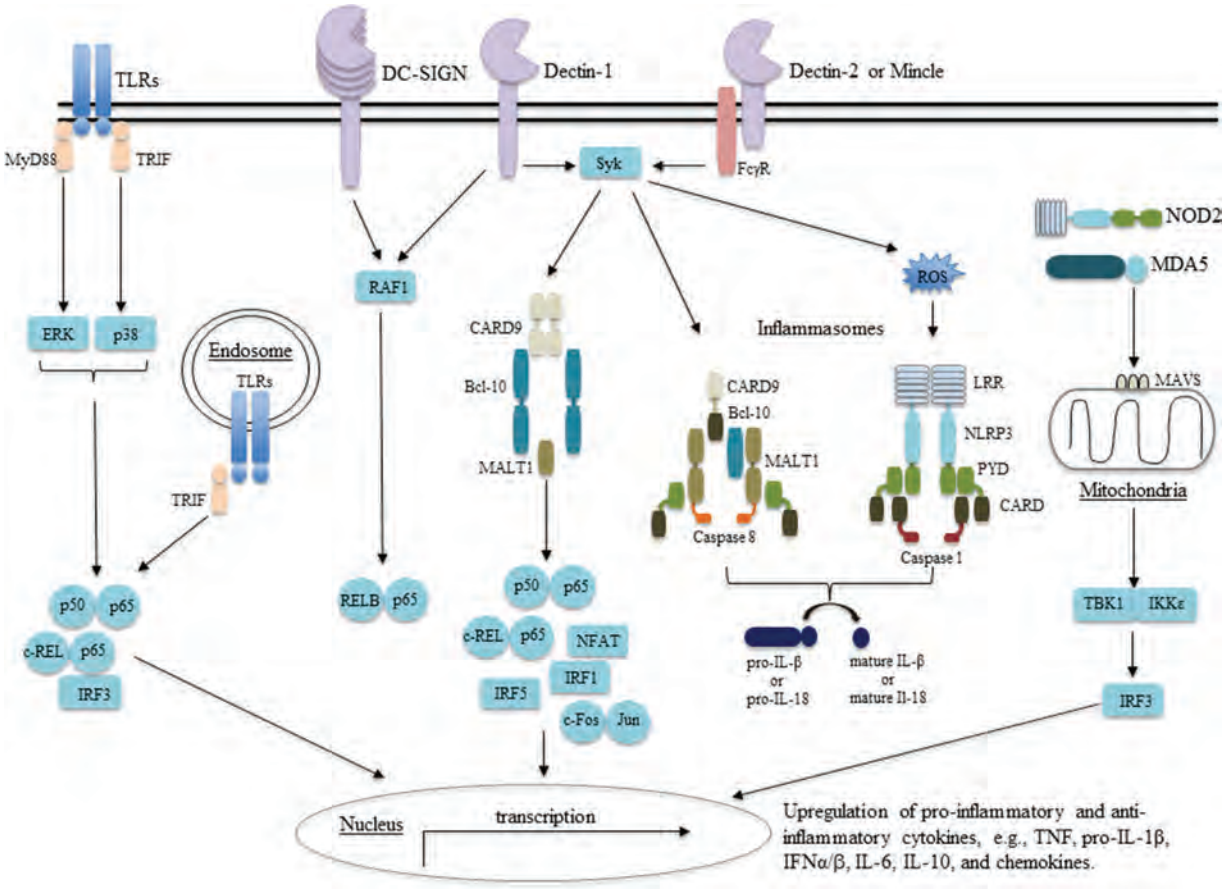


Figure 2 Signaling pathways involved in antifungal immunity.

into subfamilies based on the nature of the NH_2-terminal domain structure (34). Of particular interest to antifungal immunity is NALP3—or NACHT (NAIP [neuronal apoptosis inhibitor], CIITA [MHC class II transcription activator], HET-E [heterokaryon incompatibility protein], and TP1 [telomerase-associated protein])–LRR (leucine-rich repeats)–PYD (pyrin domains)-containing protein 3—which activates a multiprotein complex known as the inflammasome by recruiting the adaptor molecule apoptosis-associated speck-like protein containing a caspase-associated recruitment domain (CARD) to the protein-protein interaction pyrin domain that subsequently activates caspase-1-mediated cleavage of the inflammatory cytokines, pro-IL-1β and pro-IL-18, to their mature forms (35–38) (Fig. 2). To this end, several studies have linked defective NALP3-mediated IL-1β release with susceptibility to several fungi including *Candida albicans* and *A. fumigatus* (39–42). Interestingly, the main component of *Cryptococcus neoformans* capsule, glucoronoxylomannan, was suggested to facilitate intracellular survival of this pathogen via inhibition of Syk-mediated NALP3 inflammasome activation (43).

RLRs are another important family of cytosolic sensors responsible for detecting double-stranded RNA and are distinct from TLR detection of endosome-associated RNA (22). Members of this family include RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). All these proteins have a central ATPase-containing DExD/H box helicase domain as a common feature (22). RIG-I and MDA5 also contain N-terminal CARD domains that facilitate downstream signaling via an adaptor molecule mitochondrial antiviral signal, resulting in activation of mitogen-activated protein kinase, NF-κB, type I IFNs (IFNα/β), and IFN-stimulated genes (44–46) (Fig. 2).

To date, MDA5 is the only member of the RLR family suggested to play a role in antifungal defense. In both mice and humans, altered expression or function of MDA5 correlated with chronic mucocutaneous candidiasis and was associated with susceptibility to systemic

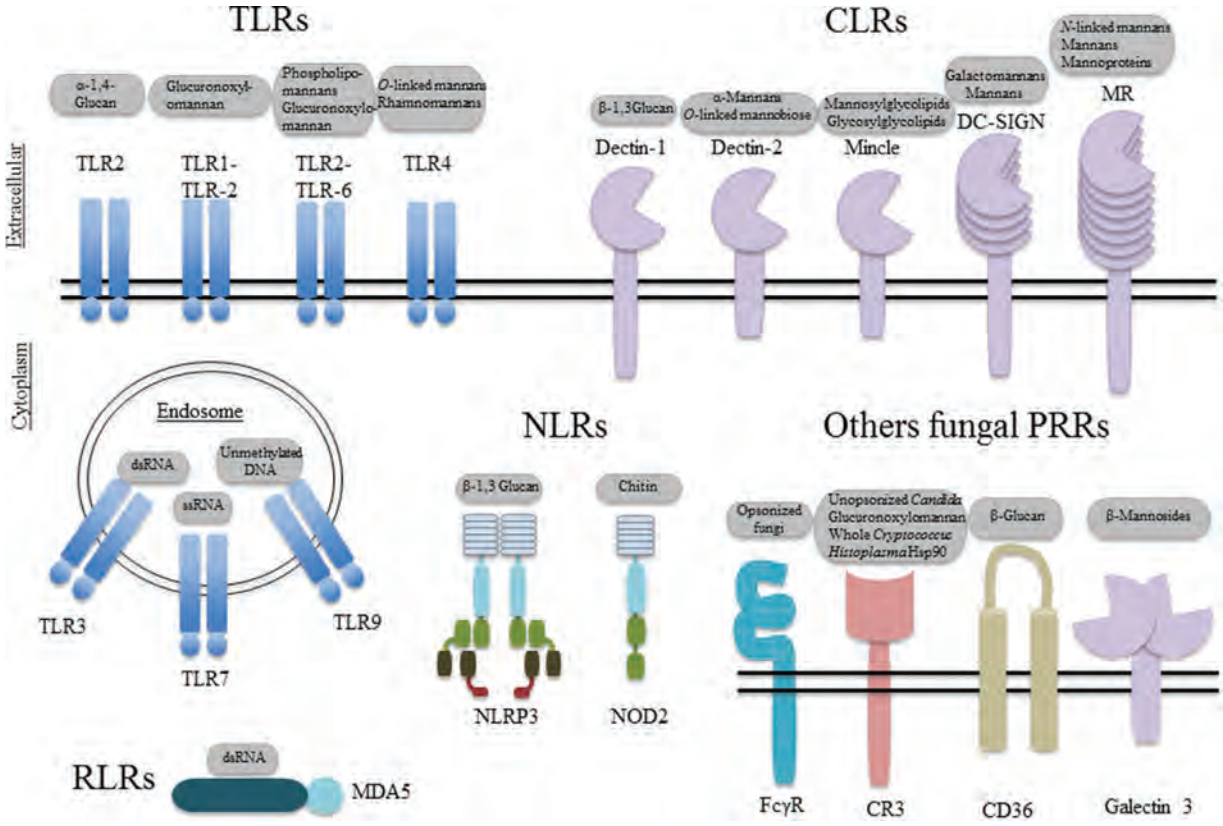


Figure 3 PRRs and the fungal components they recognize. (Adapted with modifications from reference 12).

Candida infections, presumably due to reduced expression of type I IFNs (47). Although the *C. albicans* ligand(s) that mediates activation of MDA5 is currently unknown, there is a possibility that fungal components gain access to the cytosol and induce immune responses, but this warrants further investigation. The role of type I IFNs, IRFs, and IFN-stimulated genes in antifungal immunity also needs more clarity. For instance, a protective role of IFNs was reported in mice and humans infected with *C. albicans* (48, 49), whereas in a different study using *Candida glabrata*, these cytokines were associated with susceptibility to infection (50).

CLRs Are Central for Recognition of Fungi and Antifungal Host Defense

CLRs constitute a superfamily of more than 1,000 soluble and membrane-bound proteins classically characterized by the presence of at least one C-type lectin domain (CTLD) and can be clustered into at least 17 subgroups based on architecture of the CTLD (51). The EPN (Glu-Pro-Asn) motif within the CTLD specifies binding to mannose, *N*-acetylglucosamine, L-fucose, and glucose, whereas QPD (Gln-Pro-Asp) confers specificity to galactose and *N*-acetylgalactosamine (52). While calcium-dependent binding of carbohydrates is commonly associated with the function of the CTLD, many CTLDs are known to bind a vast array of non-carbohydrate ligands including proteins, lipids, and lipoproteins (51). Of note are membrane CLRs expressed on myeloid cells that function as PRRs and prime the immune system such as the dendritic cell-associated C-type lectin 1 (Dectin-1, also known as CLEC7A), discovered in 2001 by Brown and Gordon as the receptor for β-1,3-glucan (a PAMP expressed in many, if not all, medically important fungi) (53).

Dectin-1 binding of β-glucan results in phosphorylation of the integral immunoreceptor tyrosine-based-like motif embedded in the cytoplasmic tail providing docking sites for the Src homology 2 domain of spleen tyrosine kinase (Syk) (54). Syk subsequently activates the signaling scaffold CARD9-Bcl-10-Malt-1 through PKCδ and a recently described complex of CARD9-H-Ras-Ras-GRF-1, which promotes nuclear translocation of several transcription factors including NF-κB,

nuclear factor and activator of transcription, IRF1, IRF5, and the activation of extracellular-regulated kinases pathway (55). In addition, unlike the Syk pathway, which activates both classical NF-κB (p65 and c-Rel) and noncanonical NF-κB (RelB), Dectin-1 also activates the Raf-1 kinase pathway that sequesters RelB by forming inactive p65/RelB dimers, thereby promoting Syk-mediated activation of p65 (56) (Fig. 2). Signaling via Dectin-1 regulates several processes including phagocytosis, autophagy, NETosis, respiratory burst, and gene expression of proinflammatory mediators including cytokines such as tumor necrosis factor (TNF), IL-23, IL-6, and chemokines (C-C motif) CCL2, CCL3, and (C-X-C motif) CXCL1 (57). Activation of the Dectin-1-Syk-CARD9 signaling pathway by fungi upregulates IL-1β and IL-18 transcripts and is implicated in "priming" of the NALP3/caspase 1 inflammasome, while reactive oxygen species, potassium efflux, and cathepsin B activate inflammasome activity (58, 59). More recently, the Dectin-1-Syk pathway was reported to activate a noncanonical inflammasome comprising MALT1-caspase 8 and apoptosis-associated speck-like protein containing a CARD domain that cleaved pro-IL-1β (60).

Several studies in mice have demonstrated a critical role of Dectin-1 in host defense against fungal infections. In mice lacking Dectin-1, a failure to sense the presence of fungi results in tissue overgrowth of *C. albicans* or *A. fumigatus* and is associated with an overall failure to induce appropriate inflammatory responses (such as IL-6 and granulocyte colony-stimulating factor [G-CSF]) that would normally result in the recruitment of monocytes and neutrophils to the site of infection (61–64). Importantly, individuals with polymorphisms in Dectin-1 that affect the ability of leukocytes to bind β-glucan display increased susceptibility to chronic mucocutaneous candidiasis, recurrent episodes of vulvovaginal candidiasis, onychomycosis, and intra-abdominal *Candida* infections and a reduced Th17 cell response (discussed below) (65).

CLRs such as Dectin-2, macrophage-inducible C-type lectin (Mincle), dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), and the mannose receptor have also been shown to elicit an immune response against fungi (66–68). Dectin-2 recognizes α-mannans from several fungi including *C. albicans*, *Histoplasma capsulatum*, and *C. neoformans*, as well as glycoproteins containing O-linked mannobiose-rich residues from *Malassezia* (69, 70). Signals transmitted via Dectin-2 activate the Syk-CARD9 pathway through association with an integral immunoreceptor tyrosine-based-bearing Fc receptor γ chain (FcRγ) adaptor molecule, which results in improved reactive oxygen species production by neutrophils, expression of IL-23p19, and IL-1β release by APCs (71, 72). Dectin-2-deficient mice display increased susceptibility to infection with *C. albicans* (Fig. 2) (73, 74). Similar to Dectin-2, Mincle recognition of glucosyl- and mannosylglycolipids from *Malassezia* and ill-defined ligands from *C. albicans* and *Fonsecaea pedrosoi* also activates the Syk-CARD9 pathway via the FcRγ adaptor (75). However, the role of Mincle in antifungal immunity may be context dependent. In a recent report, interaction of Mincle with the causative agent of chromoblastomycosis, *Fonsecaea monophora*, was shown to suppress IL-12p35 (an important cytokine required for T helper cell polarization) via Syk-CARD9 activation of E3 ubiquitin ligase Mdm2-dependent degradation of the transcription factor IRF-1 (76).

DC-SIGN and the mannose receptor recognize branched *N*-linked mannans and have been implicated in trafficking of mannosylated fungal antigens into the APC endocytic pathway, facilitating antigen processing and presentation to T helper cells (77, 78). In mice, a lack of mannose receptor was shown to result in susceptibility to infection with *C. neoformans* but not *C. albicans* (79, 80).

Although mouse models and *in vitro* studies with human leukocytes provide experimental support for a role in immunity of CLRs, mentioned above, to date, only polymorphisms in Dectin-1 and mutations in the signaling adaptor molecule CARD9 (which acts downstream of Syk-coupled CLRs) have been associated with susceptibility to fungal infections in humans. While polymorphisms in Dectin-1 were predominantly linked with superficial infections, CARD9 deficiency in both mice and humans was found to be critical for protection against systemic *Candida* and *Exophiala* infection, particularly of the central nervous system (49, 65, 81–83). CARD9-mediated protection against *Candida* spinal osteomyelitis and meningoencephalitis was shown to require neutrophil chemotaxis into the central nervous system (49, 65, 81, 84, 85).

EFFECTOR MECHANISMS DRIVING HOST PROTECTION FROM FUNGI

Epithelium

Apart from the obvious physical barrier function, the epithelium, through secretion of factors such as mucins and antimicrobial peptides, plays an important role in protection against microbial tissue invasion (86). Salivary mucins in host defense and disease prevention

were shown to repress formation of *C. albicans* hyphae (a morphological form thought to be associated with host invasion) and displayed direct candidacidal activities in the oral cavity, respectively (87).

Although research into "self versus nonself" interactions has for the most part focused on pathogens, commensal microorganisms, including the mycobiota (fungal communities), colonize mucosal surfaces such as the gut, and their role in health and disease is slowly gaining attention (88). For instance, increased *Candida* gut colonization and differential fungal profiles were observed in patients with inflammatory bowel disease and in ulcerative colitis patients, respectively (89–91). Notably, in these studies, an absence of Dectin-1 signaling correlated with severe forms of ulcerative colitis in both mice and humans. Intriguingly, Dectin-1 and CARD9 have also been implicated in regulation of intestinal homeostasis by modulating gut microbiota. Dectin-1-mediated induction of antimicrobial peptides S100A8 and S100A9 paralleled decreases in *Lactobacilli* numbers; these are commensal gut bacteria best known for metabolizing tryptophan into aryl hydrocarbon receptor ligands and generation of regulatory helper CD4 T cells (discussed below) (92, 93). By contrast, gut microbiota from CARD9-deficient mice failed to metabolize tryptophan and consequently displayed decreased aryl hydrocarbon receptor ligands and IL-22 and increased sensitivity to colitis (94).

Phagocytes

Derived from the Greek term *phagein*, meaning "to eat," phagocytes are critical for clearing foreign bodies and debris from dead or dying cells. Tissue-resident macrophages and neutrophils are key phagocytic cells that mediate host protection against fungi (95). Here, a few examples of the mechanisms deployed by phagocytic cells in antifungal immunity are described. Upon sensing fungi, macrophages recruit other immune cells to sites of infection through secretion of proinflammatory cytokines and chemokines, discussed above (95). *In vivo* depletion of macrophages or deficiency of CX_3C-chemokine receptor 1 (CX_3CR1), which is associated with poor survival of macrophages, was observed to result in unrestricted fungal growth in tissues and heightened mortality rates in mice systemically challenged with *C. albicans* (96). Importantly, patients with decreased expression of CX_3CR1 are highly susceptible to disseminated candidiasis (96). In the lung, alveolar macrophages kill inhaled *A. fumigatus* conidia and *Pneumocystis carinii* cysts through mechanisms involving reactive oxidant intermediates (97, 98). As immunologists increasingly define functional "subsets" of cells, specific populations of monocytes and macrophages that protect against fungi are being described. For instance, several studies have reported a protective role of $CCR2^{+}Ly6^{hi}$ inflammatory monocytes in systemic candidiasis (99, 100). In mice, depletion of this subset resulted in lethal invasive aspergillosis (101).

The importance of neutrophils is underscored by the fact that neutropenia is one of the major risk factors driving disseminated fungal infections in mouse models and in humans (95, 102, 103). Neutrophils accomplish their potent fungicidal activities through generation of reactive oxygen radicals and nonoxidative effector mechanisms including secretion of lysozyme, lactoferrin, elastase, β-defensin, gelatinases, and cathepsin G (104). Neutrophil extracellular traps, released during the last stages of neutrophil active cell death, known as NETosis, have been implicated as another strategy of antifungal defense (105–107). Neutrophil extracellular trap formation coincides with neutrophil degranulation and release of microbicidal factors such as cathelicidin (108). Proteinase 3-mediated cleavage of cathelicidin into LL-37 was shown to interfere with fungal cell membranes, suppress formation of biofilms and fungal attachment, induce reactive oxygen species production, enhance chemotaxis, and suppress neutrophil apoptosis (109–114).

Reinforcing Effectors: Cytokines

The basics of the immune process as we currently understand it were discussed in the preceding sections; that is, PAMPs are recognized by PRRs expressed by innate immunocytes, resulting in activation of the innate immune system. As described above, the bridge between innate and adaptive host immunity is mediated by professional APCs, dendritic cells, discovered in 1973 by Steinman and Cohn (115). Dendritic cells provide *signal 1* (antigen-presentation) and *signal 2* (costimulation) to naive T helper CD4 cells (bearing αβ T cell receptors) initiating "effector" antigen-specific helper CD4 T cell responses (116, 117). Other aspects including generation of cytotoxic CD8 T cells and B cell development into immunoglobulin-producing plasma cells are also activated but will be not be discussed here. In the last section, we discuss the nature of helper cells induced upon fungal recognition and the type of help they provide (Fig. 4).

Cytokines produced by APCs in response to PAMPs provide *signal 3i*, which instructs polarity of T cells (Th) into functionally distinct helper subsets. Members of the IL-12 family of cytokines are known critical mediators of Th polarization, and these include IL-12 (IL-12p35:IL-12p40), IL-23 (IL-12p19:IL-12p40), IL-27

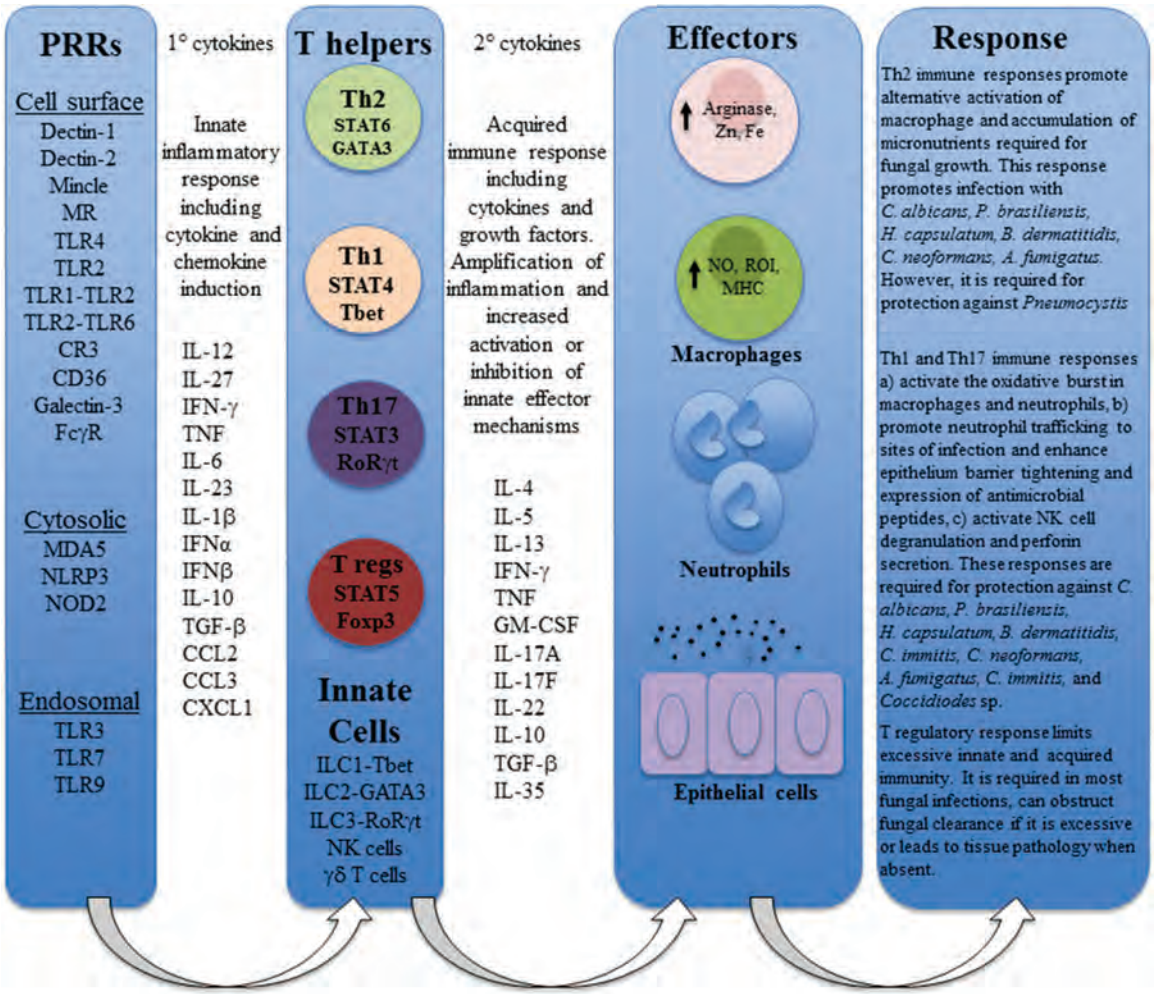


Figure 4 Schematic representation of the sequential inflammatory immune reaction involved in antifungal immune responses.

(IL-12p28:EBi3), and IL-35 (IL-12p35:EBi3) (118). Binding of these cytokines to their cell surface receptors activates the Janus kinase and signal transducer and activator of transcription (STAT) signaling pathways, trans-activating gene expression, and in the case of naive T cells, differentiation (119).

IL-12 and IL-27 secretion by innate immune cells as well as innate lymphocyte-derived IFN-γ skew naive cells toward a Th1 differentiation program driven by STAT1, STAT4, and T box transcription factor T-bet (120, 121). Classically, Th1 cells are defined by production of mainly IFN-γ but also produce other important proinflammatory cytokines such as TNF and GM-CSF. The role of Th1 cells in protective immunity against most fungal pathogens in both experimental mouse models and humans is well established. Several laboratories have shown that IFN-γ and TNF stimulate macrophages to release nitric oxide and reactive oxygen species, causing intracellular growth arrest of several fungal species such as *H. capsulatum* (122–124). An absence of Th1 immunity or expression of the effector cytokines as demonstrated in IL-12, IFN-γ, or TNF deficiency in both mice and humans results in increased susceptibility to a myriad of fungal infections, including *C. neoformans*. Patients on IFN-γ immunotherapy show augmented protection against aspergillosis, cryptococcosis, and coccidioidomycosis (125). GM-CSF, on the other hand, was shown to mediate antifungal responses in macrophages by a mechanism involving sequestering intracellular zinc (a micronutrient required by yeast cells), stimulating reactive oxygen species, as well as neutrophil recruitment (126, 127). In contrast, several laboratories reported that STAT1 (signal transducer of IL-27, type I IFNs, and IFN-γ) gain of function

mutations in humans resulted in autosomal dominant chronic mucocutaneous candidiasis, potentially due to excess induction of type 1 IFNs which promote IL-27 and IL-10, resulting in inhibition IL-17-producing T cells (128, 129) (Table 1).

It is now well established that IL-17-producing T cells play a significant role in the clearance of fungi, particularly at the mucosae. This lineage differs from Th1 in that it requires STAT3 activation by IL-6 and IL-21 as well as transforming growth factor β induction of retinoid-related orphan receptor γt, while IL-23 is required for stabilization of the phenotype (130). Effector cytokines, particularly IL-17 and IL-22, were shown to promote the release of various antimicrobial peptides by keratinocytes and epithelial cells as well as strengthening of the mucosal barrier preventing disease dissemination (131–134). The importance of this lineage is made obvious by the heightened susceptibility to a wide range of fungal infections in individuals with loss of function mutations in STAT3 or defects in the IL-17 signaling axis (135–138). In addition, mutations in the autoimmune regulator gene that result in antibodies targeted at Th17 cytokines predispose to chronic and recurrent mucocutaneous candidiasis as noted in individuals with autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy (APECED) (125). Decreased antimicrobial peptide secretion as well as reduced neutrophil-attracting CXC chemokines due to a lack of Th17-mediated immune responses are thought to be key drivers of susceptibility to mucosal fungal infections (135, 138).

Polarization toward the Th2 subset requires IL-4-mediated STAT6 activation and GATA3 expression, which drive induction of the signature cytokines: IL-4, IL-5, and IL-13. Th2-cytokines are considered important for promoting alternatively activated macrophages reported to permit intracellular growth of fungi due to decreased nitric oxide expression (139). In mouse models, a pathogenic role of an elevated Th2 immune response is best exemplified by the observation of progressive lung infections with *C. neoformans* and *H. capsulatum* (69).

Regulatory T cells (T regs) are unique helper cells that play the essential role of restraining immune responses limiting collateral damage to the host. Mechanisms of action mediated by T regs include secretion of inhibitory cytokines such as IL-10, transforming growth factor β, IL-27, IL-35, contact-dependent mechanisms, and sequestration of IL-2 (125). Decreased T reg numbers as observed in TLR2- or CCR5-deficient mice are thought to drive efficient clearance of several fungal infections including *C. albicans* (140, 141). Interestingly, defective natural T reg development is associated with pathogenesis of chronic mucocutaneous candidiasis in APECED patients, highlighting the importance of the delicate balance between host-driven immune pathology and resolution of infection (142).

Table 1 Selected human genetic associations with fungal susceptibility discussed in this article

Gene	Immunological phenotype	Associated disease
Dectin-1	Dimunition of cytokine responses	Chronic mucocutaneous candidiasis, vulvovaginal candidiasis, onychomycosis, and intra-abdominal *Candida*
CARD9	Dimunition of cytokine responses Defective neutrophil chemotaxis	Disseminated candidiasis
TLR1	Decreased IL-1β, IL-6, and IL-18	Candidemia
TLR2	Decreased IFN-γ and IL-8	Candidemia
TLR3	Reduced activation of NF-κB and decreased IFN-γ	Cutaneous candidiasis
NALP3	Decreased IL-1β?	Candidiasis-mediated vestibulitis
MDA5	Decreased type I IFNs	Systemic candidiasis
STAT1	Decreased IL-17 production, elevated cell response to IFNs, IFN-γ,s and IL-27 Defective IL-12R and IL-23R signaling	Chronic mucocutaneous candidiasis, coccidioidomycosis, histoplasmosis
STAT3	Defective Th17 polarization, decreased IL-17/IL-22-expressing T cells, decreased IL-17-induced antimicrobial peptides	Chronic mucocutaneous candidiasis, coccidioidomycosis, histoplasmosis, cryptococcosis, aspergillosis, nail dermatophytosis
Autoimmune regulator	Neutralizing antibodies to IL-17 and IL-22	Chronic mucocutaneous candidiasis
IL-17/IL-17R	Lack of IL-17 cellular responses	Chronic mucocutaneous candidiasis
IL-12RA	Impaired IL-12 and IL-23-mediated expression of IFN-γ	Coccidioidomycosis, paracoccidioidomycosis, histoplasmosis, cryptococcosis

AIDS and susceptibility to fungal infections such as *Cryptococcus*, *Candida*, *Pneumocystis*, and *Histoplasma* have focused attention on the critical contribution of T cells to the host defense arsenal against fungal pathogens (143). Yet cells of the innate immune system such as natural killer cells, γδ T cells, and the recently described innate lymphoid cells also contribute to the effector cytokine pool, and the contribution of these cells in antifungal defense is under intense investigation (133). A case in point is work from LeinbundGut-Landmann's laboratory demonstrating that antibody depletion of innate lymphoid cells in Rag-deficient mice (which lack mature classical T helper cells) resulted in attenuation of IL-17 and increased susceptibility to oropharyngeal candidiasis infection (144). Moreover, this group also reported that IL-17-mediated immune responses are critical for natural killer cell activation and secretion of GM-SCF, which is essential for antifungal immunity (145). Another study reported natural killer cell direct killing of *C. neoformans* and *Cryptococcus gattii* through mechanisms that involved perforin (146). The contribution of these cells to invasive fungal disease and their relevance in human disease are still under investigation.

IMMUNE-BASED THERAPIES AGAINST FUNGAL INFECTIONS

Vaccines

A major hurdle in this area has been largely impacted by the lack of understanding of the requirements for induction of protective immunity and defining surrogate markers for these responses. This is made apparent by the lack of any fungal human vaccine approved for clinical use to date. However, there are exciting developments including studies showing that mice immunized with β1-3-D-glucan-laminarin-diphtheria toxoid conjugate vaccine or antigens encapsulated with glucan particles induce a strong antibody response and a long-term antifungal T cell response that protects mice against several fungal species including *Cryptococcus* and *Candida* (147, 148). The use of a live vaccine strategy has also shown promising results in several settings. Examples such as immunization with a *C. neoformans*-expressing IFN-γ strain offer advantages for developing therapeutic vaccines that offer protection in conditions where CD4 helper T cells are deficient (149).

Adaptive Cell Therapies

Fungal-specific T cell transfer studies have been shown to improve disease outcomes in high-risk individuals such as hematopoietic transplant patients by providing enhanced control of *Aspergillus* antigenemia and reducing the mortality rate (150). More recently, T cells bearing modified chimeric antigen receptors have been investigated for potential use in fungal diseases (for details about chimeric antigen receptor technology see reference 151). In a proof-of-concept study, T cells bearing Dectin-1 (directing T cell specificity to fungal cell wall glucans) were shown to mediate protection against *A. fumigatus* (152).

As discussed above, neutropenia is associated with susceptibility to fungal infections, and approaches that reduce the duration of neutropenia in chemotherapy patients have obvious attractive therapeutic implications. Thus, using G-CSF and GM-CSF to increase the yield of granulocytes from donors is gaining a lot of interest in clinical settings where granulocyte transfusions are frequently used as supportive therapy (153). Another exciting approach is immune modulation via exogenous provision of costimulation to effectively activate the immune responses. This was demonstrated by a recent study showing that chronicity of chromoblastomycosis caused by *F. pedrosoi* was due to lack of costimulation with TLRs; thus, topical application of TLR ligands such as imiquimod (which signals via TLR7) resulted in an adequate inflammatory response that resolved the infection (154).

FUTURE PERSPECTIVES

In the 27 years since the inception of the PRR theory, much progress has been accomplished. We now know that there are other pathogen-sensing mechanisms exemplified by ideas such as the guard theory, which posits that the mammalian immune system detects cellular processes that are targeted by the pathogen's virulence factors. Furthermore, identification of the cell biology components such as compartmentalization of sensing receptors into membrane-bound, cytosolic, or endosomal modules has emerged as an important framework for the activity of the innate immune system. Understanding how all of these pathways are integrated and controlled from different compartments in the context of fungal infections will be essential for advancing our understanding of antifungal immune responses and developing future immunotherapies. Moreover, additional efforts using a systems biology approach to better define immunological, cellular, and molecular pathways involved in antifungal responses at different anatomical sites will also expand our knowledge. Studies characterizing the complex interplay between host genetics, microbiota, and the virome

in health and disease will help decipher an age-old question: what is the basis for mammalian protective immunity?

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Antifungal Drugs: The Current Armamentarium and Development of New Agents

44

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INTRODUCTION

Importance of Fungal Pathogens

Disease and death caused by fungal infections have transitioned from a rare curiosity to a major global health problem. Because the vast majority of fungi capable of causing life-threatening infections target individuals with impaired immunity, recent decades have witnessed a stark rise in fungal infections due to medical interventions such as chemotherapy for cancer and immunosuppression for organ transplantation and due to the prevalence of HIV infection (1). Pathogenic fungi are the causative agents of billions of infections annually, with ~1.5 million attributable mortalities (2). The public health burden is comparable to that observed with more notable infectious diseases such as tuberculosis and malaria, yet the impact of fungal infections on human health has been largely overlooked (3).

The predominant fungal pathogens of humans include *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. *C. albicans* is a common component of the human mucosal microbiota and is responsible for millions of superficial infections in immunocompetent individuals, such as thrush and oropharyngeal candidiasis. It is also the most prevalent fungal etiological agent of life-threatening invasive infections, with mortality rates approaching 40% despite treatment (1). The CDC has classified *Candida* species as a serious threat to human health due to the dramatic rise in resistance to antifungal drugs (4). Cryptococcosis caused by *C. neoformans* and the closely related species *Cryptococcus gattii* affects over a million individuals annually, with mortality rates as high as 70% in Latin America and sub-Saharan Africa (2). Recently, *C. gattii* was associated with outbreaks of cryptococcosis in healthy individuals on Vancouver Island (5) and other regions of the Pacific Northwest (6), making *Cryptococcus* a public health threat to immunocompromised and immunocompetent individuals. Finally, *A. fumigatus* is a significant cause of invasive infections in individuals with impaired immune function, including those with neutropenia, solid organ transplant recipients, and patients on immunosuppressive therapies such as high-dose corticosteroids (2). It is estimated that more than 200,000 cases of invasive aspergillosis occur each year, with staggering mortality rates of up to 50% with treatment and 100% if left undiagnosed (2). Thus, these chief opportunistic invaders are a leading cause of human mortality and impose a dire need for effective antifungal therapeutics to combat these infections.

In this article we aim to describe the current armamentarium of antifungal agents and the most promising emerging therapies in the antifungal development pipeline. We also highlight the innovative strategies being leveraged to identify novel antifungal targets and uncover new therapeutic strategies to combat fungal infectious disease.

Challenges for Treatment of Fungal Infections

Fungi are eukaryotic, like their human hosts, limiting the number of molecular targets that can be selectively exploited for drug development without the risk of cross-target toxicity. The current arsenal of antifungal compounds approved for the treatment of invasive infections consists of only three classes of antifungal agents

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directed against a limited number of cellular processes. Perhaps of greater concern is that there are few discovery programs devoted to the development of new antifungal therapeutics because of the expectation of limited financial return for pharmaceutical companies (2). Most antifungals target ergosterol, the functional fungal analogue of cholesterol; the biosynthesis of ergosterol; or the biosynthesis of (1,3)-β-D-glucan, a major component of the fungal cell wall (Fig. 1). This paucity of fungus-specific targets is problematic because of the prevalence of cross-resistance to all drugs with a common target. Resistance to all widely deployed antifungals has been documented in both the laboratory and the clinic (7) and continues to be a growing problem in the medical community. There is a pressing need to bolster the antifungal pipeline, as highlighted by the Generating Antibiotic Incentives Now (GAIN) Act (H.R. 2182), which promises a major effort to reduce the incidence of drug-resistant fungi and bacteria in the United States through antimicrobial discovery.

CLASSES OF ANTIFUNGAL DRUGS IN CLINICAL USE

Polyenes

Since the 1950s, the fungicidal polyenes have served as a powerful but highly toxic last line of defense for combating invasive fungal infections. Polyenes are amphipathic, consisting of a hydrophobic polyene hydrocarbon chain and a hydrophilic polyhydroxyl chain. For decades the prevailing theory was that these molecules elicited their toxic effects by intercalating into ergosterol-containing membranes to form membrane-spanning channels that cause leakage of cellular components and, ultimately, cell death (8, 9). However, recent detailed structural and biophysical studies highlighted that polyenes act more like an "ergosterol-sponge," forming large extramembranous aggregates that extract the essential membrane-lipid ergosterol from the plasma membrane (Fig. 1) (10). Given that polyfunctional lipids such as ergosterol serve as central molecular nodes in cellular physiology and function, resistance to the polyenes is strongly evaded and comes at a significant fitness cost to the fungus (11). The differential binding affinity of amphotericin B for ergosterol over cholesterol, the mammalian membrane sterol, accounts for its selectivity; however, amphotericin B does affect mammalian cells, and nephrotoxicity is a common side effect of clinical usage that can be mitigated somewhat by liposomal formulations (8). Despite these drawbacks, amphotericin B is still used for the treatment of the most serious fungal infections, in large part due to its broad spectrum of activity.

Pyrimidines

Pyrimidines such as flucytosine (5-fluorocytosine) were first used to treat fungal infections in the 1960s. However, the rapid onset of resistance precludes the use of flucytosine as a monotherapy, and consequently it is only used in combination with other antifungals such as amphotericin B. This drug combination remains the "gold standard" for treating *Cryptococcus* infections and other less common invasive mycoses (12). Flucytosine is a synthetic fluorinated analogue of cytosine. Upon entry into the cytosol, flucytosine becomes rapidly deaminated to generate 5-fluorouracil by fungal specific cytosine deaminases (13). 5-Fluorouracil acts as a potent antimetabolite that causes RNA miscoding and inhibits DNA synthesis (Fig. 1).

Azoles

The azole antifungals encompass compounds with both imidazole and triazole moieties. They are the most widely deployed class of antifungal in clinical use because of their impressive safety profile and availability in both oral and intravenous formulations. For example, oral azoles are more broadly accessible in resource-limited settings such that oral fluconazole is widely used to treat cryptococcal meningitis in these regions despite treatment outcomes being much better with the i.v.-administered combination of amphotericin B and flucytosine (14). Furthermore, voriconazole is the drug of choice for aspergillosis based on its superiority to amphotericin B in a head-to-head clinical trial (15, 16). The azoles consist of a five-membered nitrogen-containing heterocyclic moiety, which binds to an iron atom in the heme group located in the active site of the drug target lanosterol 14-α demethylase (also referred to as cytochrome $P450_{14DM}$ and Erg11) (Fig. 1). Azole binding blocks the demethylation of lanosterol, resulting in ergosterol depletion and the accumulation of toxic sterol intermediates, which ultimately impedes cellular growth and division and induces severe membrane stress (7, 8). Most of the antifungal imidazoles are formulated for topical use, as a consequence of toxicity or bioavailability problems that limit their potential as systemic agents. The only agents licensed for treatment of invasive fungal disease are triazoles and include fluconazole, itraconazole, posaconazole, voriconazole, and isavuconazole (8, 17). The azoles are typically fungistatic, with the notable exception of voriconazole, which has fungicidal activity against *A. fumigatus*. The fungistatic nature of azoles imposes strong directional

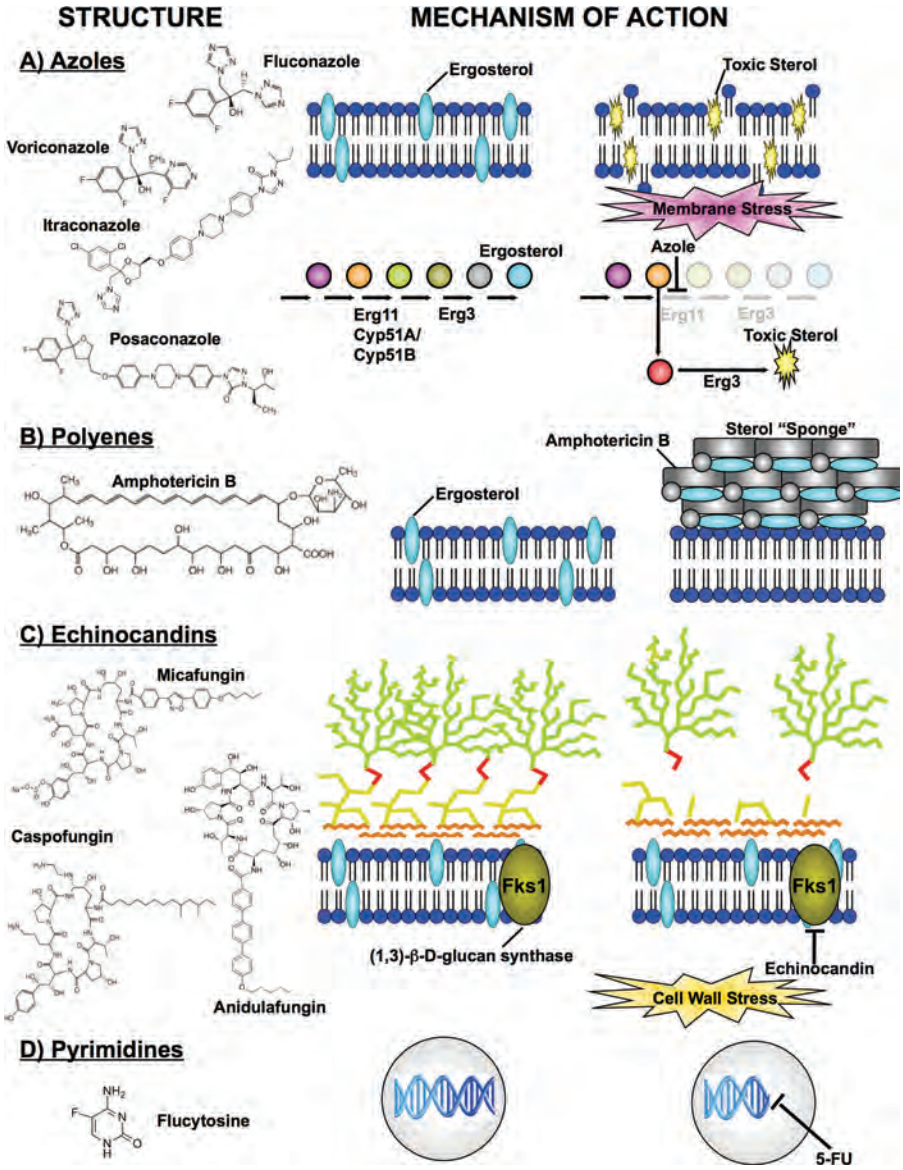


Figure 1 Structures and mechanisms of action of clinically relevant antifungal drugs. (**A**) The azoles function by targeting the ergosterol biosynthetic enzyme lanosterol demethylase, encoded by *ERG11* (*C. albicans* and *C. neoformans*) or *cyp51A* and *cyp51B* (*A. fumigatus*), causing a block in the production of ergosterol and the accumulation of a toxic sterol produced by Erg3. This toxic sterol exerts a severe membrane stress on the cell. (**B**) Polyenes such as amphotericin B primarily exist in the form of large extramembranous aggregates that extract ergosterol from lipid bilayers. (**C**) Fungal cell walls are composed of (1,3)-β-D-glucans covalently linked to (1,6)-β-D-glucans as well as chitin, mannans, and cell wall proteins. The echinocandins act as noncompetitive inhibitors of (1,3)-β-D-glucan synthase (encoded by *FKS1* in *C. albicans*, *C. neoformans*, and *A. fumigatus*) and thereby cause a loss of cell wall integrity and severe cell wall stress. (**D**) Pyrimidines such as flucytosine become rapidly deaminated in the cytosol to generate 5-fluorouracil (5-FU) by fungal-specific cytosine deaminases. 5-FU acts as a potent antimetabolite that causes RNA miscoding and inhibits DNA synthesis. Adapted from reference 185 with permission.

selection for the evolution of resistance. Moreover, several *Candida* species such as *Candida glabrata* and *Candida krusei* are intrinsically less susceptible to this class of drugs.

Echinocandins

The echinocandins are the only new class of antifungal to reach the clinic in decades, with three echinocandins currently available for clinical use: caspofungin, micafungin, and anidulafungin. These compounds are cyclic hexapeptides that act as noncompetitive inhibitors of (1,3)-β-D-glucan synthase, an enzyme involved in cell wall synthesis (Fig. 1). (1,3)-β-D-Glucan is an essential cell wall polysaccharide that provides structural integrity. Inhibition of (1,3)-β-D-glucan synthesis culminates in a loss of cell wall integrity and severe cell wall stress (18). The safety profile of these compounds is impressive and likely attributable to a fungal-specific target that is not conserved in mammals. Despite their potent activity against *Candida* and *Aspergillus*, these compounds are completely ineffective at combating *Cryptococcus* infections (18). Echinocandins have emerged as an important treatment option for candidiasis, especially in the context of the increasing prevalence of azole resistance.

DEVELOPMENT OF IMPROVED AGENTS WITHIN EXISTING CLASSES

Polyenes

In an attempt to improve the therapeutic index of amphotericin B, alternative lipid-associated formulations have been developed and incorporated into clinical use since the 1990s (19). Amphotericin B lipid complex (ABLC) consists of amphotericin B in a complex with two lipids: L-α-dimyristoyl phosphatidylcholine and L-α-dimyristoyl phosphatidylglycerol (19). The large molecular size of the compound results in rapid uptake of the drug by macrophages, which distribute the compound throughout the mononuclear phagocyte system including target organs such as the liver and spleen (19). Consequently, compared with the conventional formulation, there are lower circulating amphotericin B serum concentrations and reduced accumulation in the kidneys. Also of benefit, ABLC fails to stimulate myriad proinflammatory signaling molecules *in vitro* (20). Liposomal amphotericin B (L-AmB), marketed as AmBisome, is composed of a small unilamellar vesicle formulation consisting of hydrogenated soy phosphatidylcholine, cholesterol, and distearoyl phosphatidylglycerol. Amphotericin B is tightly bound to the liposome through charge pairing between the amino group of the amphotericin B and the phosphate group of distearoyl phosphatidylglycerol. Tissue concentrations in patients receiving L-AmB tend to be highest in the liver and spleen and much lower in the kidneys and lung. In a rabbit model of hematogenous *C. albicans* meningoencephalitis, L-AmB accumulates in the brain at higher levels than standard amphotericin B, significantly decreasing fungal burden in this tissue (21). This supports the preference for L-AmB in treatment of invasive central nervous system infections. Amphotericin B colloidal dispersion, marketed as Amphocil and Amphotec, encompasses two molecules of amphotericin B bound to two molecules of cholesteryl sulfate. Similar to the other liposomal formulations, there is reduced drug accumulation in the kidneys. However, unlike the other liposomal formulations, amphotericin B colloidal dispersion induces a high degree of inflammatory gene upregulation (20), which was likely a contributing factor related to the adverse side effects observed in several clinical studies (22, 23).

In addition to these lipid polyene formulations, alternative preparations continue to be developed. A cochleate formulation of amphotericin B displayed efficacy in experimental models of candidiasis and aspergillosis (24–26). Cochleates are a novel lipid-based delivery vehicle consisting of crystalline phospholipid-cation structures that form spiral lipid sheets. They represent a new technology platform for oral delivery of clinically important drugs that possess poor oral bioavailability. Initial biodistribution studies of cochleate amphotericin B administered orally in mice found that cochleates deliver significant levels of amphotericin B to target organs (24, 26). Despite these successes in animal models, clinical trials conducted by BioDelivery Sciences International appear to have been discontinued.

For the treatment of most invasive fungal infections, an amphotericin B lipid formulation provides a safer alternative than conventional amphotericin B, especially in the context of nephrotoxicity. Unfortunately, despite their use in the clinic for close to 20 years, it is difficult to conclude which (if any) of these lipid formulations possesses superior *in vivo* antifungal activity compared to amphotericin B or compared to each other due to a paucity of well-designed, randomized trials and also due to the multitude of invasive infections caused by evolutionarily diverse fungal pathogens.

Due to the pharmacological importance of amphotericin B in treating systemic mycoses, medicinal chemists have made extensive efforts to generate amphotericin B derivatives with more favorable pharmacological

properties and reduced toxicity. Modification of the mycosamine appendage through a double reductive alkylation generated a semisynthetic molecule with improved efficacy against amphotericin B-resistant *C. albicans in vitro* and reduced hemotoxicity (27). Improvements to the therapeutic index of amphotericin have also been made in a C2′deoxyamphotericin B derivative (28). Often the major limitation of these derivatives is the limited synthetic access, which precludes future development. Recently, synthetic chemists reported urea derivatives of amphotericin B that are easily accessible from the natural product by using a three-step synthesis reaction (29). These derivatives bind ergosterol with far greater selectivity than amphotericin B *in vitro* and maintain potent antifungal activity with reduced toxicity in mouse models of disseminated candidiasis (29). Moreover, urea-containing amphotericin B derivatives strongly evade resistance (29). The promising biological activities coupled with favorable synthetic properties suggest bright prospects for these amphotericin B derivatives in combating invasive fungal disease.

Azoles

Given that azoles are the most widely deployed class of antifungal, there is a long history of developing novel analogues with improved efficacy and safety. Isavuconazole was developed as an orally active, water-soluble prodrug that is also amenable to intravenous formulations (Fig. 2). The prodrug, termed BAL-8557, becomes cleaved in a reaction catalyzed by plasma esterases in mammalian cells (30). It exhibits a long elimination half-life, has broad-spectrum activity against *Candida* and *Aspergillus* species, and has demonstrated good efficacy in several clinical trials (31). One such study supported the safety and tolerability of isavuconazole as a prophylactic treatment in immunosuppressed patients at high risk of fungal infections (32). This compound has undergone phase III clinical trials to evaluate efficacy in the treatment of *Aspergillus* infections in renally impaired patients and those infected with other rare fungi (clinicaltrials.gov NCT00634049), as well as to compare efficacy to a caspofungin-voriconazole cotherapy against *Candida* infections (NCT00444366 and NCT00413218). In 2015, isavuconazole was approved by the U.S. Food and Drug Administration (FDA) for the treatment of invasive *Aspergillosis* and invasive mucormycosis.

Albaconazole is another triazole currently under development with potent broad-spectrum antifungal activity, good pharmacokinetics, and excellent oral bioavailability (Fig. 2). A phase II clinical study evaluated the therapeutic effects of a single dose of albaconazole in women with acute *Candida* vulvovaginitis and found that doses of ≥40 mg had greater therapeutic effects than fluconazole at 150 mg (30). Despite the efficacy in treatment of vulvovaginitis, it remains unclear whether albaconazole will have utility in treatment of invasive fungal infections.

Echinocandins

The echinocandins were first introduced into the clinic in 2001. Since this time there has been incentive to develop novel analogues in this class, primarily to overcome the current limitation of echinocandins to daily intravenous dosing regimens. Aminocandin (Fig. 2), also referred to as IP960 or HMR3270, is a semisynthetic fermentation product from *Aspergillus sydowii*. Its long half-life makes infrequent dosing a feasible option, and it has shown promising *in vitro* and *in vivo* activity against *Candida* and *Aspergillus* species (33, 34). Similarly, Cidara Therapeutics has developed an echinocandin derivative, CD 101 IV, which shows a dramatically extended half-life in animal models of *C. albicans* infection (35, 36). Preclinical evaluations in rats uncovered no hepatotoxicity at doses up to 20 mg/kg for 2 weeks (37). Currently, this compound is in phase I clinical trials (NCT02551549) and has been designated a qualified infectious disease product with fast track status from the FDA.

Enfumafungin is a structurally distinct natural product class that is similar to the echinocandins in targeting glucan synthase (38). The current development candidate MK-3118 is an orally active, semisynthetic derivative of enfumafungin with potent *in vitro* activity against glucan synthase and potent *in vivo* activity against *Candida* and *Aspergillus* species (Fig. 2) (39, 40). Thus, novel echinocandin derivatives and distinct antifungals targeting glucan synthase activity represent promising new treatment options to combat *Candida* and *Aspergillus* infections.

WHAT CHEMICAL MATTER SHOULD WE BE THINKING ABOUT FOR NEW DRUGS?

The optimal chemical space for antifungal compounds is poorly defined. Unlike antibacterial drugs that are dominated by microbial natural products, antifungals include both synthetic and natural product compounds in equal proportion (Fig. 1). In fact, synthetic compounds dominate the fungicides used in agriculture. This should be good news for antifungal drug discovery because, in contrast to antibacterial campaigns, existing libraries of synthetic compounds in pharmaceutical

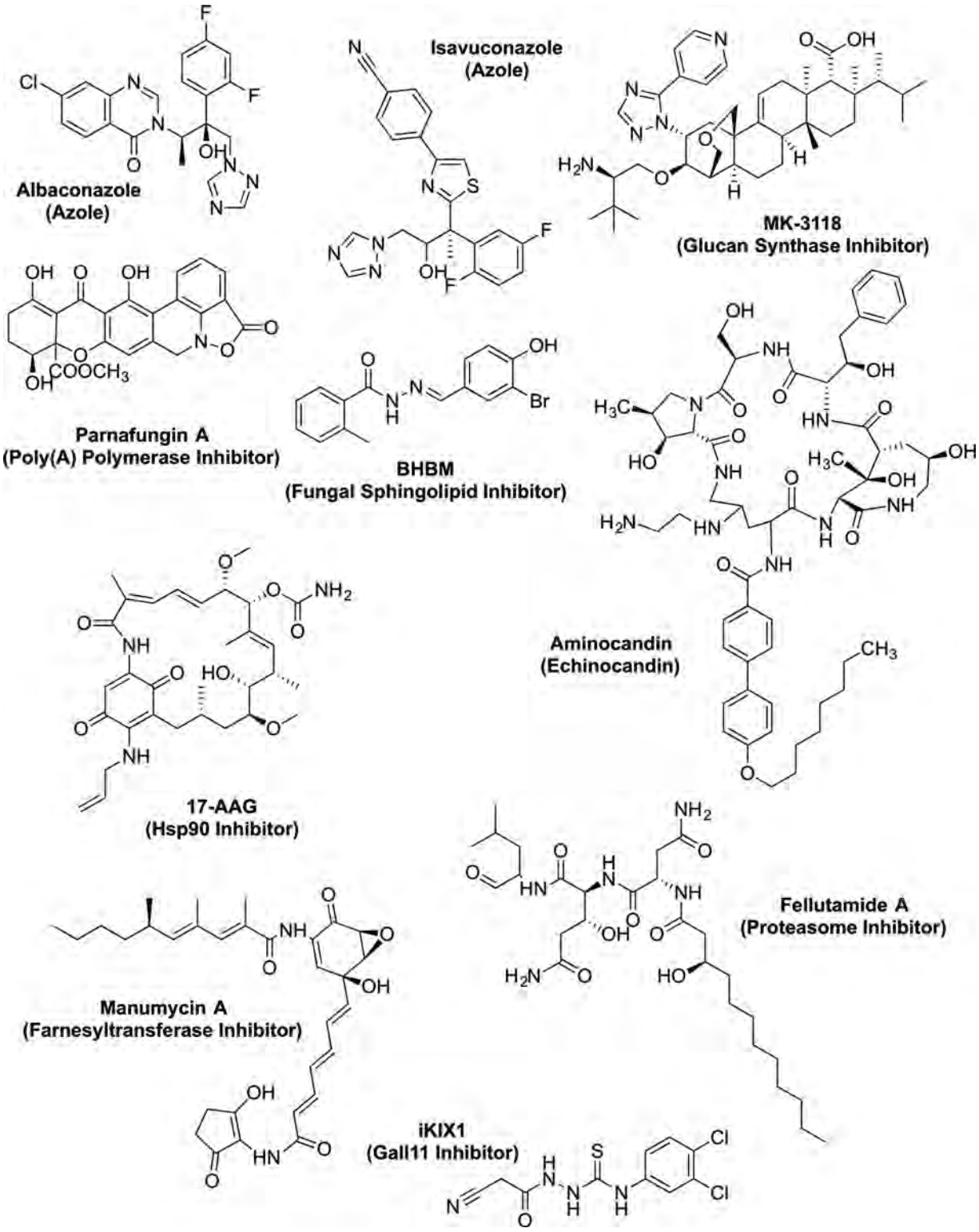


Figure 2 Structures of compounds with antifungal activity. Chemical structures of antifungal molecules highlighted throughout the review. The description in brackets describes the molecular target of the chemical compound.

companies are likely to be good starting points for antifungal screens. There are challenges in using these compounds, however. For example, there are fungal-specific PAIN (pan assay interference) compounds such as the rhodanines that are promiscuous in antifungal campaigns but have a poor likelihood of generating good leads in drug development (41). Additionally, the synthetic compounds that dominate pharmaceutical company libraries are enriched in molecules with physicochemico characteristics such as the Lipinski rule of five (42), which emphasize human targeted drugs. As a result, compounds identified in antifungal screens from such libraries often overlap with compounds that have bioactivity against human cellular targets. This reflects the relatively narrow unique target space between fungi and host biology (human, animal, or plant). Threading the needle between uniquely antifungal targets and the host is significantly challenging, especially if screened compounds are biased toward human biology. Indeed, as noted below, many highly useful drugs for human diseases, such as rapamycin, were first identified in antifungal screens. Not surprisingly then, most antifungal drugs have significant off-target effects that are reflected in host toxicity.

Expanding screens of synthetic molecules to libraries that typically have not yielded bioactivity in human targets (so-called chemical dark matter) can also offer an entry point into a selective antifungal target space. A group at Novartis specifically mined such chemical dark matter in a screen for antifungal compounds that identified a new compound and a new target, heme biosynthesis (43).

Another option is a return to screens of natural products to identify new leads in antifungal drug discovery and development. Screens of microbial natural product extracts often return polyenes such as amphotericin, but these can often be quickly dereplicated based on their characteristic absorption spectra. The Merck group has been especially active in this area over the past decade, developing new screening technologies (44) and reporting a series of novel natural products with antifungal activity that modulate new targets such as poly(A) polymerase (45) and the fungal proteasome (46).

The narrow target space for antifungal drugs, the diversity of fungal physiology, and the absence of systematic physicochemico rules for nontoxic antifungal agents complicate antifungal drug discovery. Nevertheless, clever screens for new targets and judicious mining of natural products and synthetic molecule libraries continue to yield new antifungal leads and offer encouragement for future success.

NEW ANTIFUNGAL DRUG TARGETS IN DEVELOPMENT

Our current arsenal of antifungal drugs target either ergosterol synthesis (azoles), ergosterol itself (polyenes), or cell wall synthesis (echinocandins). These targets account for a minor fraction of the potential drug targets encoded by genomes of fungal pathogens (47). Here we highlight some of the most promising new antifungal drug targets currently being explored for development of novel therapeutics.

Fungal Sphingolipids

Sphingolipids are ubiquitous eukaryotic membrane components that play a central role in signal transduction, cell regulation, and virulence in pathogenic fungi (48). There are a variety of fungal-specific sphingolipids present in the cell membrane and cell wall including glucosylceramides (GlcCer), which are glycosphingolipids composed of a glucose unit bound via a glycosyl linkage to a ceramide moiety. As proof-of-principle, genetic studies have established that targeting sphingolipid biosynthesis holds promise for blocking fungal virulence. In *C. neoformans*, strains lacking GlcCer synthase 1 are avirulent in a mouse inhalation infection model (49). Similarly, deletion of genes required for sphingolipid biosynthesis in *C. albicans* renders the pathogen avirulent (50). These genetic studies predict that small molecules targeting sphingolipid biosynthesis and/or function may serve as novel antifungal agents.

High-throughput screening efforts have been fruitful in identifying small molecules that target sphingolipid biosynthesis. A screen of 49,120 small molecules to identify inhibitors of fungal sphingolipid GlcCer synthesis identified two compounds: [*N′*-(3-bromo-4-hydroxybenzylidene)-2-methylbenzohydrazide (BHBM) (Fig. 2) and 3-bromo-*N′*-(3-bromo- 4-hydroxybenzylidene) benzohydrazide (D0)] (51). BHBM and D0 were efficacious against *C. neoformans in vitro*, in an intranasal infection model, and in an invasive infection model of the central nervous system (51). Paradoxically, although BHBM and D0 did not show potent antifungal activity against *C. albicans in vitro*, they were efficacious in a model of invasive candidiasis (51). Genome sequencing of drug-resistant mutants identified four candidate targets of BHBM: Apl5, Cos111, Mkk1, and Ste2, which are involved in vesicular transport and cell cycle progression (51). Thus, this study has identified promising compounds that inhibit fungal GlcCer.

Beyond small molecules, additional strategies to target sphingolipid biosynthesis show considerable promise. Monoclonal antibodies specific to *C. neoformans* GlcCer provide protection against *C. neoformans* infec-

tion, likely due to downregulation of the inflammatory response (52). Moreover, treatment with Cerezyme, a recombinant enzyme that metabolizes GlcCer, hydrolyzes fungal GlcCer in growing *C. neoformans* cultures, disrupts membrane integrity, and exhibits *in vivo* efficacy in an inhalation murine model of cryptococcosis (53). Notably, sphingolipid biosynthesis is also important for the host. Compromised sphingolipid biosynthesis in mice confers hypersensitivity to *C. albicans* infection due to the inability of immune cells to phagocytose yeast and clear the infection (54). These studies reinforce the potential of sphingolipids as an antifungal drug target and highlight the importance of specifically targeting fungal, and not mammalian, sphingolipids.

GPI Anchor Biosynthesis

Many cell surface proteins are modified at their C-terminus by the addition of a glycosylphosphatidylinositol (GPI) anchor, which consists of a lipid group, myoinositol, glucosamine, several mannose residues, and a phosphoethanolamine group that connects the GPI anchor to the protein through an amide bond (55). This posttranslational modification results in proteins being transported from the endoplasmic reticulum (ER; the site of modification) to the plasma membrane and cell wall, where they are directly or indirectly involved in cell wall organization. GPI anchoring is a conserved posttranslational modification in eukaryotes, although the number of mannose groups and the position of side chains on the GPI anchors vary widely between species. In *C. albicans*, deletion of *GPI7*, a gene with important roles in GPI anchor synthesis, results in abnormal morphogenesis and reduced virulence (56). In *A. fumigatus*, transcriptional repression of genes involved in GPI-anchor biosynthesis results in cell wall defects, abnormal morphology, attenuated virulence, and increased cell death through autophagy and necrosis (57, 58). Further, the GPI-anchored protein Ecm33 has important roles in *A. fumigatus* morphogenesis, cell wall stress response, and virulence (59).

Multiple small molecules that inhibit GPI-anchor biosynthesis have been discovered. The first was identified by a screen for molecules that interfere with the cell wall localization of a GPI-anchored reporter protein (60). The initial hit was 1-[4-butylbenzyl] isoquinolone, a molecule shown to target the acyl transferase Gwt1 (60). Medicinal chemical-based optimization led to development of the pyridine-2-amine-based molecule E1210, a compound with potent antifungal activity against numerous pathogenic fungi (61). Subsequently, a small molecule originally identified as an inducer of ER stress, gepinacin, was determined to be a specific inhibitor of Gwt1, a critical acyltransferase required for the biosynthesis of fungal GPI anchors (62). Sublethal concentrations of gepinacin or genetic disruption of GPI-anchored proteins exposes elevated levels of (1,3)-β-D-glucan on the cell surface, enhancing the host immune response against *C. albicans* (62, 63).

Farnesyltransferase

Farnesyltranferase is a ubiquitous eukaryotic enzyme that catalyzes posttranslational lipidation on the C-terminus of more than 120 important signaling proteins in mammalian cells (64). This acts as a signal, which culminates in the translocation of proteins from the ER to the plasma membrane. Protein substrates of farnesyltransferase bear a C-terminal CAAX sequence motif: cysteine (C), two aliphatic residues (AA), and a variable (X) residue. Ras proteins are key modulators of diverse signaling cascades in eukaryotes and are an example of proteins modified by farnesyltransferases. In pathogenic fungi, Ras signaling pathways regulate virulence, morphogenesis, and cell cycle control (65, 66). In the model yeast *Saccharomyces cerevisiae*, deletion of the gene *RAM1* encoding farnesyltransferase results in reduced fitness, but it is not essential for viability (67). However, farnesyltransferases are essential for growth and virulence in *C. neoformans*, *C. albicans*, and *A. fumigatus* (68–70). Farnesyltransferase inhibitors such as manumycin A (Fig. 2), originally developed as an anticancer therapeutic, have antifungal activity against *C. neoformans* and block localization of *C. neoformans* Ras1 to the cell membrane (71). Manumycin A also displays some activity against *A. fumigatus* and *C. albicans* (72). Additional farnesyltransferase inhibitors, such as ethylenediamine inhibitor #2 and tipifarnib, also show some antifungal activity against *C. neoformans*, particularly in a mutant deficient in capsule production (71). Finally, farnesyltransferase inhibitor III blocks the *C. albicans* morphological transition from yeast to hyphae, a virulence trait regulated by Ras signaling (73).

Key physical interactions between farnesyltransferases and their inhibitors have been illuminated through structural studies comparing fungal farnesyltransferases with their human homologs. Using X-ray crystallography, conformational differences between these enzymes from humans and *C. neoformans* have been identified, supporting feasibility of the development of fungal-selective farnesyltransferase inhibitors (71). Similarly, structural studies of farnesyltransferase enzymes from *A. fumigatus* confirmed structural differences from the human enzyme that are sufficient for species-specific targeting (74). Although much of the work with

farnesyltransferase inhibitors has focused on their impacts on Ras localization and signaling, mapping the fungal farnesylome may also uncover novel drug targets for future antifungal development. One example is the Ras-related protein RhbA in *A. fumigatus*, which is important for fungal pathogenesis and vegetative growth *in vitro* and for virulence in an *in vivo* model of infection (75).

Calcineurin

A principal regulator of cellular stress responses in all eukaryotes is the Ca^{2+}-calmodulin-activated protein phosphatase calcineurin (76, 77). In pathogenic fungi, calcineurin regulates myriad physiological processes including cell cycle progression, cation homeostasis, morphogenesis, virulence, and antifungal drug responses (76, 77). Calcineurin is required for survival at physiological temperatures in *C. neoformans* but is dispensable for growth in *C. albicans* and *A. fumigatus* (77). Hence, the calcineurin signaling pathway has a conserved role in fungal biology and virulence yet also possesses unique specializations across the fungal genera.

Pharmacological inhibitors of calcineurin function, including the natural products cyclosporin A and tacrolimus, have potential therapeutic indications. For example, these agents are deployed in the clinic as immunosuppressants to prevent graft rejection in transplant recipients (77). Their immunosuppressive activity is due to inhibition of calcineurin-dependent activation of a key transcriptional regulator termed the nuclear factor of activated T cells (NFAT), which is involved in T cell activation. In fungi, calcineurin inhibitors have diverse effects on cellular stress responses and virulence. Pharmacological inhibition of calcineurin in *C. neoformans* abrogates growth at 37°C (78), potentiates the activity of both azoles and echinocandins in *C. albicans* (79–83), and renders the fungistatic azoles fungicidal against multiple *Candida* species (84). Further, cyclosporin A and tacrolimus act synergistically with azoles against *C. albicans* biofilms both *in vitro* and *in vivo*, implicating calcineurin as a key modulator of azole resistance during biofilm growth (85). In *A. fumigatus*, calcineurin inhibitors enhance echinocandin activity and transform the fungistatic activity of caspofungin to fungicidal (86, 87). Calcineurin inhibitors also show potent activity against azole- and echinocandin-resistant strains of *A. fumigatus* (88). Recently, it was shown that calcineurin orchestrates dimorphic transitions, antifungal drug responses, and virulence of the fungal pathogen *Mucor circinelloides* (89, 90). Thus, targeting calcineurin provides a logical strategy for combating fungal disease caused by evolutionarily diverse fungi.

Therapeutic exploitation of calcineurin as a target for antifungal drug development requires fungal-selective agents. Nonimmunosuppressive cyclosporin A and tacrolimus derivatives retain antifungal activity against *C. neoformans*, including azole-resistant strains (91). These nonimmunosuppressive analogues also retain synergistic activity with azoles against *C. albicans* (84). Chromofungin, a synthetic vasostatin-I-related peptide, acts as a calcineurin inhibitor and also displays *in vitro* activity against *A. fumigatus* (92). Development of effective antifungal therapeutic agents that target calcineurin signaling will require crystal structures of calcineurin from fungal pathogens coupled with structure-guided drug design to enhance potency and fungal-selectivity of calcineurin inhibitors or development of agents targeting fungal-specific calcineurin effectors (77).

Hsp90

Hsp90 is an essential molecular chaperone that regulates the form and function of diverse client proteins (93, 94). As a consequence of its role in stabilizing key signal transducers, Hsp90 modulates the relationship between genotype and phenotype by acting as both a capacitor and a potentiator for the storage and release of genetic variation (95–97). In pathogenic fungi, targeting Hsp90 function as an antifungal strategy holds considerable promise given that it orchestrates crucial cellular responses to drug-induced stress (98–100). In *S. cerevisiae* and *C. albicans*, genetic depletion or pharmacological inhibition of Hsp90 impairs the evolution of azole resistance and abrogates resistance that has been acquired through diverse molecular mechanisms (82, 83). Compromise of Hsp90 function also transforms azoles from fungistatic to fungicidal (82). Pharmacological inhibition of Hsp90 in *C. neoformans* and *C. gattii* inhibits growth and potentiates azole activity *in vitro* (101). Moreover, in *C. albicans*, *C. glabrata*, and *A. fumigatus*, Hsp90 enables basal tolerance and resistance to the echinocandins. Thus, Hsp90 has a profound impact on resistance to multiple antifungal classes in diverse fungal pathogens (81, 102–104).

Hsp90 regulates responses to antifungal-induced stress through its downstream effectors calcineurin and protein kinase C signaling (81, 105). In a series of clinical isolates recovered over time from an HIV-infected patient treated with fluconazole, the basal fluconazole resistance phenotypes of isolates recovered early during treatment were dependent on Hsp90, calcineurin, and Pkc1 function (82, 105). This dependence gradually evolved toward independence of Hsp90, calcineurin, and Pkc1, corresponding with upregulation of multi-

drug transporters (82, 105, 106). Biochemical analysis confirmed that Hsp90 stabilizes the catalytic subunit of calcineurin and the terminal MAP kinase of the Pkc1 cell wall integrity pathway, Mkc1 (81, 105). Hsp90 is one of the most highly connected hubs in cellular networks, interacting with an estimated 10% of the yeast proteome (107, 108). Chemical genomic studies in *C. albicans* identified a multitude of Hsp90 genetic interactors important for cellular responses to azoles and echinocandins (109), suggesting that Hsp90 may have pleiotropic effects on circuitry governing drug resistance.

Hsp90 is also a powerful therapeutic target given its function in orchestrating central virulence traits such as fungal morphogenesis. In *C. albicans* the ability to transition between distinct morphological states is correlated with virulence (50) and is tightly regulated by diverse environmental cues coupled with elevated temperatures of at least 37°C (7). Genetic or pharmacological inhibition of Hsp90 function induces filamentation by relieving its repression on Ras1-protein kinase A signaling (110, 111). Elevated temperatures similarly relieve Hsp90-mediated repression of filamentation as the chaperone becomes overwhelmed with global protein misfolding in the cell (110, 112). To add additional complexity, Hsp90 also regulates *C. albicans* morphogenesis through the cyclin Pcl1, the cyclin-dependent kinases Pho85 and Cdc28, and the transcription factor Hms1 (113, 114). Intriguingly, genetic depletion of Hsp90 in *A. fumigatus* has the opposite effect in that it decreases hyphal growth and leads to severe defects in germination and conidiation (102). Given its intimate and complex role in governing fungal morphogenesis in multiple species, targeting Hsp90 holds promise as an antivirulence strategy to combat fungal infection.

The impact of Hsp90 inhibition on fungal virulence and antifungal efficacy has been explored using a variety of *in vivo* infection models. In *Galleria mellonella* models of fungal pathogenesis, combination therapy with Hsp90 inhibitors that have been in clinical development for cancer, such as 17-AAG (Fig. 2), with either caspofungin or fluconazole improves survival upon infection with *A. fumigatus* or *C. albicans*, respectively (115). Furthermore, in murine models of *C. albicans* disseminated disease, genetic compromise of fungal *HSP90* expression reduces virulence (110) and enhances the therapeutic efficacy of fluconazole and caspofungin (81, 115). Similarly, genetic compromise of *A. fumigatus* Hsp90 abrogates virulence in a murine model of invasive aspergillosis (104). In a *Caenorhabditis elegans* model of cryptococcal infection, Hsp90 inhibition enhances the activity of fluconazole (101). Finally, in an *in vivo* rat catheter model of infection, 17-AAG transforms fluconazole from ineffective to highly efficacious without host toxicity in the context of this localized infection and drug delivery (116). In the context of systemic fungal infections, toxicity associated with inhibition of host Hsp90 precludes the use of current Hsp90 inhibitors as antifungals and demands the development of fungal-selective Hsp90 inhibitors or agents that target fungal-specific regulators of Hsp90 function.

Acetyltransferases and Deacetylases

Lysine deacetylases (KDACs) and lysine acetyltransferases (KATs) catalyze the removal or addition of acetyl groups from the ε-amino group of lysines. Such modifications in core histones result in changes in chromatin structure and gene expression (117, 118). Consequently, KDACs and KATs have pleiotropic effects on cellular signaling and stress responses in fungi. The broad-spectrum KDAC inhibitor trichostatin A potentiates azole activity in *C. albicans* (119, 120). Further, *C. albicans* clinical isolates are sensitive to nicotinamide, an inhibitor of NAD^+-dependent KDAC complexes, and nicotinamide reduces fungal kidney burden in a mouse model of disseminated candidiasis (121). Nicotinamide is also active against other *Candida* species and against *A. fumigatus* and *Aspergillus nidulans*, suggesting broad antifungal properties (121). Further, a molecule thought to inhibit the KDAC Hos2, MGCD290 (Mirati Therapeutics, San Diego, CA), displays synergistic activity with azoles and echinocandins against diverse *C. albicans* drug-resistant clinical isolates (122, 123). These studies were restricted to *in vitro* analysis, but *in vivo* studies coupled with preliminary clinical trials have supported the use of MGCD290 in combination with fluconazole to treat *C. albicans* infections (124). Thus, these findings suggest considerable promise for the use of KDAC inhibitors in combination with current antifungals in combating invasive fungal disease.

In addition to their roles in modifying histones, KDACs also deacetylate a spectrum of nonhistone proteins, such as Hsp90. Acetylation of Hsp90 provides a mechanism of posttranslational control that governs the emergence and maintenance of Hsp90-dependent azole resistance (120). In *S. cerevisiae*, Hsp90 is acetylated on lysine 27 and 270, residues that are regulated by KDACs Rpd3 and Hda1 (120). Genetic or pharmacological inhibition of both Hda1 and Rpd3 blocks the physical interaction between Hsp90 and calcineurin, thereby impeding calcineurin function (120). Deletion of both *HDA1* and *RPD3* does not increase global Hsp90 acetylation levels, suggesting that additional

KDACs orchestrate Hsp90 deacetylation. One candidate KDAC is Hos2, which interacts genetically with Hsp90 under diverse environmental conditions in *C. albicans* (109). In *A. fumigatus*, Hsp90 lysine residues important for drug resistance and virulence are conserved with those identified in *S. cerevisiae* (120, 125). Thus, by influencing gene expression through histone modification and controlling the function of key signal transducers, KDAC inhibitors hold promise as antifungal therapeutics.

KATs also modulate azole resistance. Genetic impairment of *C. albicans ADA2*, which encodes a component of the Spt-Ada-Gcn5-acetyltransferase (SAGA) coactivator complex, confers hypersensitivity to fluconazole due to impaired upregulation of the efflux pumps Cdr1 and Mdr1 (126). Further, deletion of the *C. albicans* KAT gene *RTT109* confers hypersusceptibility to macrophages, altered metabolic gene expression, and induction of a weaker inflammatory response, culminating in attenuated virulence in a murine infection model (121, 127). Deletion of *RTT109* also confers increased sensitivity to echinocandins and to other genotoxic stresses such as hydroxyurea and methyl methane sulfonate (121).

Other Antifungal Targets

The TOR protein kinases are global regulators of cellular growth in response to nutrient cues, including amino acids (128). In *S. cerevisiae*, inhibition of TOR signaling has a multitude of effects including induction of autophagy, inhibition of translation, and repression of ribosomal gene expression, as well as upregulation of genes involved in the retrograde response, nitrogen catabolite response, and stress response (128–130). TOR can be inhibited pharmacologically by the immunosuppressant rapamycin (sirolimus). Rapamycin is a natural product of the bacterium *Streptomyces hygroscopicus*, originally identified in a screen for antimicrobial activity against *C. albicans* and later found to have potent immunosuppressive activity (131). Pioneering genetic studies in *S. cerevisiae* identified FKBP12, Tor1, and Tor2 as the direct targets of rapamycin (132). Rapamycin inhibits growth of diverse fungi, including *C. neoformans*, *C. albicans*, *A. fumigatus*, *Fusarium oxysporum*, and *Penicillium* spp. (133, 134) and abrogates *erg3*-mediated azole resistance (135). Analogues of rapamycin with reduced immunosuppressive activity retain antifungal activity *in vitro* (136).

There are several other antifungal agents in development that target a variety of other cellular processes. Parnafungins inhibit poly(A) polymerase and display potent broad-spectrum activity against clinically relevant *Candida* species and therapeutic efficacy in murine models of candidiasis (Fig. 2) (45). Inhibitors of leucine tRNA synthases, such as tavaborole (AN2690), are highly selective against *Trichophyton* species (137, 138) and are in clinical development to treat onychomycosis (14). Finally, inhibitors targeting the 26S proteasome (fellutamides, Fig. 2), translation elongation (yefafungin), cyclic AMP homeostasis (campafungin), and microtubule dynamics (12-deoxy-hamigerone) are all unexploited areas that may represent the next breakthrough in novel antifungal scaffold (14, 44, 46). While it is impossible to predict which, if any, of the targets highlighted above will prove to be the next clinically exploited antifungal target, the portfolio of distinct prospects provides renewed hope for the development of novel antifungal treatments.

COMBINATION THERAPY TO EXPAND THE REPERTOIRE OF DRUG TARGETS

Although advances are being made to improve our current antifungal arsenal and to identify novel drug targets, the rate at which antimicrobials are being produced is vastly outpaced by the rate at which resistance is emerging (139, 140). A promising strategy for combating drug-resistant fungi is through combination therapy. Drug combination therapy has benefits such as enhanced efficacy and specificity when compared to individual drug treatments and can impede the evolution of resistance (141, 142). Further, by carefully selecting specific drug combinations, microbial drug resistance may be not only neutralized but also reversed through a process called selection inversion (143). Combination therapy provides the foundation for treatment of other infectious diseases such as HIV (144), tuberculosis (145), and malaria (146). Despite the therapeutic potential, there is only one combination that has been well validated in clinical trials for fungal infections: the combination of amphotericin B and flucytocine as the gold standard to treat cryptococcosis (12). Combination therapy also has the potential to unveil myriad additional antifungal targets given that fungal biology is controlled by a highly interconnected and functionally redundant network of biological interactions (147, 148). The yeast deletion project revealed that only ~1,000 of the ~6,000 genes in the *S. cerevisiae* genome are essential for viability (149). However, a tour de force systematic analysis of ~5.4 million pairs of gene deletions identified ~170,000 negative genetic interactions, including 10,000 synthetic lethal interactions (148). Synthetic lethality represents an extreme negative genetic interaction in which two mutations, causing little to no fitness defect individually, result in a lethal

double mutant phenotype (150). The potential for harnessing synthetic lethality provides a new avenue for antimicrobial drug discovery through the use of combination agents.

There is precedent for leveraging the power of genetic interactions to predict combination drug therapy. In *S. cerevisiae*, *FKS1* and its paralog *FKS2* encode the biosynthetic enzyme for (1,3)-β-D-glucan synthesis and the molecular target of the echinocandins. *FKS1* and *FKS2* are synthetically lethal, in keeping with echinocandin efficacy. *FKS1* is also synthetically lethal with *CHS3*, a chitin synthase required for the synthesis of the cell wall component chitin (148), and inhibitors of chitin synthases, such as nikkomycin, are synergistic with caspofungin against numerous fungal pathogens (151, 152). *FKS1* is also synthetically lethal with *CNB1*, the regulatory subunit with calcineurin, and as highlighted above, calcineurin inhibitors exert potent synergy with echinocandins against diverse fungi (77). While these examples support the fact that genetic interactions underpin the network response to compound combinations, the complexities of chemical action and genetic network density seem to preclude the prediction of synergism on a genome-wide scale (153). Thus, additional factors must be employed to identify effective antifungal combinations.

In addition to leveraging genetic interaction landscapes to predict chemical synergy, target-specific inhibitors may be screened *ad hoc* for synergistic activity against specific antifungal agents. We explored many of these examples above, as with the capacity of inhibitors of Hsp90 (98, 99), calcineurin (77), KDACs (120, 123), and TOR (136), to potentiate antifungals against fungal pathogens. Other notable examples include potentiation of azole activity by pharmacological inhibition of ADP-ribosylation factors (154), protein kinase C (105), and protein translation (155). Further, tamoxifen and other triphenylethylene-based estrogen receptors have antifungal activity against *C. neoformans* (156) and potentiate the activity of azoles, polyenes, and echinocandins in diverse fungal species (157). Tamoxifen inhibits the *C. neoformans* calmodulin protein Cam1, which activates calcineurin (158). Given that Cam1 is fungal-specific, optimization of the triphenylethylene scaffold provides a promising path for antifungal drug development.

Small molecules that target resistance determinants can be effectively paired with currently available antimicrobials to combat drug-resistant isolates. Examples of such drug combinations in the clinic include drugs such as amoxicillin, a β-lactam antibiotic, with clavulanic acid, an inhibitor of β-lactamase drug-resistant enzymes (140). A recent study with *C. glabrata* identified a small molecule (iKIX1) (Fig. 2) that inhibits azole efflux pump expression by disrupting the interaction between the mediator complex and the transcriptional activator Pdr1 (159). Follow-up *in vivo* studies supported the use of iKIX1 to potentiate fluconazole efficacy against fluconazole-resistant *C. glabrata* in both metazoan and mammalian models of infection (159).

A complementary approach to identify synergistic drug interactions employed in both academia and industry utilizes high-throughput screening of large compound collections. Molecules that enhance the activity of established antifungals against *S. cerevisiae* have been identified by chemical biology screens (160, 161) and computational methods (162–164). Recent large-scale screening efforts have extended the strategy of chemical synthetic lethality to the fungal pathogens *C. albicans* and *C. neoformans*. Two such studies examined the potential of drugs approved for other therapeutic indications to potentiate current antifungal agents (157, 165). Compounds that increase azole efficacy were found to often target membrane function or sphingolipid biosynthesis (165). One such example is the antimycobacterium compound clofazimine, which induces cell membrane stress in fungi (157). A combination of clofazimine and caspofungin was efficacious *in vivo* against *C. albicans* in a *G. mellonella* model of infection (157). These studies illustrate the potential of combining current antifungal treatments with repurposed medications.

CHEMICAL GENOMICS TO LINK BIOACTIVES TO CELLULAR TARGETS

A variety of genetic and genomic approaches have been developed to uncover the molecular target for a compound whose mechanism of action remains elusive. Traditionally, forward chemical-genetic approaches have been used to identify targets by selection of resistant mutants coupled with plasmid-based library complementation, as well as gene and whole-genome sequencing (166).

More recently, reverse chemical-genetic approaches have become widely used to identify the mechanism of action of bioactive agents. In *S. cerevisiae*, haploinsufficiency profiling screens were developed to identify targets of bioactive compounds *de novo* based on the principle that reducing the dosage of a drug target confers hypersensitivity to the drug. In these assays, a genome-scale collection of heterozygous deletion strains are pooled, grown in the presence of compound, and sampled over time (167, 168). Each heterozygous

strain has a unique barcode sequence that enables deconvolution of strain identity in pooled screens (169). Quantitative analysis of strain fitness can be achieved by next-generation sequencing or microarray technology to monitor barcode abundance (167, 168). Homozygous deletion profiling (HOP) provides a complementary assay to investigate the mode of action of compounds that lack an essential protein target (for example, DNA or lipids) or for those compounds with redundant protein targets (166). Further, HOP reveals the genes important for buffering the cellular response to a drug. For example, compounds that target DNA generate HOP profiles that identify nonessential DNA-repair genes and complexes required for surviving DNA damage (170).

Large chemogenomic screening efforts have unveiled chemical-genetic interaction maps for thousands of small molecules and revealed how eukaryotic cells respond to cellular perturbations. An intensive study with 1,144 haploinsufficiency profiling and 418 HOP experiments identified growth phenotypes for mutants covering 97% of the *S. cerevisiae* genome (171). This expands our knowledge of yeast physiology and suggests that most genes are conditionally essential with functions that can be probed by small molecules. Chemogenomic profiles generated for 3,250 compounds in *S. cerevisiae* revealed that the cellular response to small molecules is largely restricted to a network of 45 chemogenomic signatures (172). These signatures highlight the fundamental small molecule response system in eukaryotic cells and provide a resource for the exploration of relationships between genes, biological processes, and chemical structures (172). Using chemical-genomic datasets, researchers recently developed a machine-learning-based prediction algorithm capable of predicting chemical synergies from large chemical libraries (153). This algorithm achieves a 6-fold enrichment in predicting synergistic chemical pairs with activity against fungi compared with chance selection alone, providing an effective platform for identifying antifungal chemical combinations.

Enabled by the development of powerful functional genomics resources, analogous tools for chemical biology are now available for the fungal pathogens *C. albicans* (173, 174) and *C. neoformans* (175). It is becoming abundantly clear that gene essentiality and chemical-genetic responses diverge rapidly over evolutionary time (175, 176), underscoring the importance of performing chemical genomic analyses directly in fungal pathogens.

In addition to chemogenomic profiling, the development of a functional variomics tool provides a complementary approach to elucidate drug mode of action (177). This technology applies plasmid-based, high-complexity random mutagenesis (or variomic) libraries covering ~90% of the *S. cerevisiae* genome. Screening this library against test compounds allows for systematic discovery of resistance-conferring mutations, thereby defining residues critical for drug binding and identifying potential mechanisms of resistance (177). Research combining chemogenomic and variomic tools has led to the identification of two novel antifungal geranylgeranyltransferase I inhibitors, highlighting the importance of an integrated platform for drug target identification (178).

OUTLOOK

It is clear that creative and focused efforts are urgently needed to bolster the antifungal pipeline and improve clinical outcome for the millions of people with life-threatening fungal infections worldwide. There are new strategies on the horizon to expand the development opportunities beyond targeting essential genes or cellular functions. A complementary approach is to target proteins that are required for pathogen growth in the host or for virulence. Identifying nonessential virulence factors opens new opportunities for chemical diversity in therapeutic drugs, because the cognate inhibitors are not typically explored by conventional screening approaches. Although the potential utility of targeting nonessential genes has only recently been appreciated, there are already examples of antibiotics that target essential structures rather than essential proteins, as with daptomycin and the Gram-positive cell wall (179). Further, the diphtheria and tetanus vaccines, as well as the antitoxin for botulinum toxin, are examples of life-saving treatments targeting nonessential virulence factors (180, 181). Targeting virulence factors offers many benefits including expanding the repertoire of antifungal targets, minimizing effects on the host mycobiome, and reducing selection pressure for the evolution of drug resistance (182, 183). With advances in genomics technologies and structure-guided drug design, as well as having antifungals now granted "orphan status" by the FDA and a priority in the GAIN act (H.R. 2182) to facilitate clinical trials (184), there is great potential to foster stronger relationships between academia and industry to accelerate antifungal drug discovery.

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Fungal Interactions with Animals (Fungi, Insects, and Nematodes) and Other Microbes

VIII

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The Insect Pathogens

45

Brian Lovett and Raymond J. St. Leger

INTRODUCTION

Estimates of the number of arthropod species vary between 1,170,000 and 10 million, accounting for over 80% of all known living animal species. One arthropod subgroup, insects, is the most species-rich member of all ecological guilds in land and freshwater environments (1). As arthropods were emerging as the dominant animals they are today, fungi were also colonizing the land. Over the past 400 million years fungi and insects have coevolved a wide array of intimate interactions (2, 3). These interactions include mutualistic endosymbiosis (4); fungi as obligate food sources, such as those found in fungus-gardening ants (5); sexually and behaviorally transmitted parasites, such as *Laboulbeniales* (6); and the most common disease-causing agents of insects (7). Entomopathogenicity has evolved independently and repeatedly in all the major phyla of the Kingdom Fungi (3). The heterogeneity of entomopathogenic fungi probably derives from both they and their hosts having short generation times, i.e., rapidly driving new diversity with each generation, and from their occupation of a wide range of habitats, with near ubiquity in the soil and on plants. Interactions among fungi, hosts, and the environment are therefore diverse and dynamic, which complicates comparisons between different fungi infecting different insects since their interactions may be necessarily disparate. Historically, this quandary was dealt with by intensively studying the host pathogen interactions of a couple of experimentally tractable fungal species, and then extrapolating these results to distantly related species. Consequently, most of what we know about the biochemical and molecular basis of interactions between fungi and insects has been determined with the experimentally tractable hypocrealean ascomycete genera *Metarhizium* (family *Clavicipitaceae*) and *Beauveria* (family *Cordycipitaceae*). *Metarhizium*, in particular, has also emerged as an excellent model to explore a broad array of questions in ecology and evolution, host preference and host switching, and the mechanisms of speciation.

Comparative genomics offers a way forward for assessing poorly characterized species. Already, comparative genomics has facilitated the identification of fungal fitness traits and the selective forces that act upon them, improving our understanding of how entomopathogenic fungi interact with insects and the environment. In particular, sequence data have provided crucial information on the poorly understood ways that these organisms reproduce and persist in the environment, identified the genes involved in ecologically relevant traits, and illuminated the nature, timing, and architecture of the genomic changes governing the origin and processes of local adaptation. Alongside the recent availability of genomic resources, the wide array of experiments that can be performed with entomopathogenic fungi makes them ideal models for answering basic questions on the genetic and genomic processes behind adaptive phenotypes (a "Holy Grail" in biology). In addition, Meyling and Hajek have described how insects and their fungal pathogens could be used as model species for exploring metapopulation theory via experimental and predictive models: consideration of the interactions insect pathogenic fungi have with their host and the broader community enables ecological questions to be probed from a unique perspective (8).

There are a lot of detailed reviews dealing with entomopathogenic fungi and their development as microbial control agents, including those from Lacey et al. (9), Vega et al. (10), and several chapters in Lovett and St. Leger (11). Relevant chapters in Roy et al. (12) detail the crucial roles fungal entomopathogens play in natural ecosystems, and for methods and techniques used to study these pathogens consult the chapters in Stock et al. (13). Studies on the evolutionary genetics of insect immunity and on insect-pathogen coevolution

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are reviewed by Hu and St. Leger (14), and entomopathogen genomes are reviewed by Wang et al. (15). This review highlights recent advances in our understanding of insect pathogenic fungi deriving in large part from a plethora of genome sequences that have illuminated the nature of adaptive differences by which novel pathogens emerge and form. These studies have increased the utility of these fungi as important model and applied organisms, allowing us to focus attention on their convergent evolution, pathogenic strategies, and host specificity, which frequently involves manipulation of host behavior.

THE UTILITY OF INSECT PATHOGENIC FUNGI

Early interest in insect pathogenic fungi grew mostly from economic concerns. For example, Agostino Bassi described *Beauveria bassiana* in 1835 as the cause of the devastating muscardine disease of silkworms, and it was instrumental in his development of the germ theory of disease (16). There is still considerable research interest in protecting beneficial insects, particularly bees, from highly potent fungal pathogens, such as the causative agent of chalkbrood, *Ascosphaera apis* (17). The research interest in insect pathogens has also been propelled by the enormous ability of insects to negatively impact human society. It is estimated that insects destroy approximately 18% of the world's annual crop production, and vector-borne diseases kill millions every year (18). Since populations of most insects are regulated by density-dependent factors, the diseases, pathogens, and immune responses of insects have been a long-standing research interest. In 1880, the pioneer immunologist Elie Metchnikoff was among the first to propose practical methods of microbial biological control of an insect crop pest, initiating trials of the fungus *Metarhizium anisopliae* against grain beetles (19). Entomopathogenic fungi are particularly well suited for development as biopesticides because, unlike bacteria and viruses that have to be ingested to elicit disease, fungi are contact insecticides and typically infect insects by directly penetrating the surface of the insect (cuticle) and multiplying in the insect body cavity (hemocoel) (Fig. 1). Approximately 170 pest control products have been developed based on at least 12 species of fungal entomopathogens (20).

Killing the host slowly is adaptive for many entomopathogenic fungi because it allows them to replicate in large numbers in the living host, but this is a severe limitation in modern agriculture or when attempting to control a disease vector. Insect pathologists have been trailblazers in utilizing genetic engineering to improve the efficacy of beneficial fungi by improving their

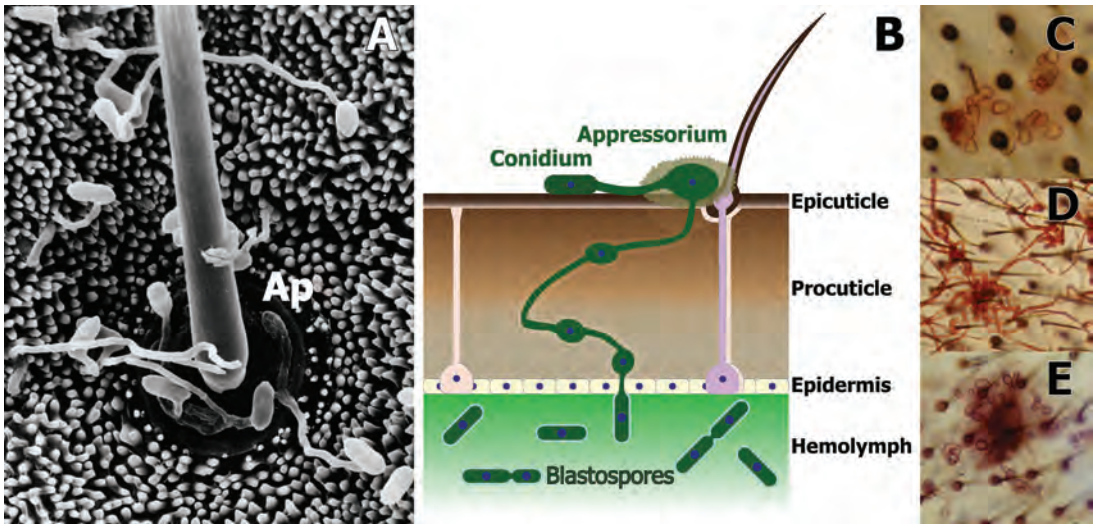


Figure 1 (**A**) Scanning electron micrograph of *M. robertsii* growing on caterpillar (*Manduca sexta*) cuticle; appressoria (Ap) were most frequently produced on zones of weakness such as hair sockets. (**B**) Diagrammatic representation of cuticle penetration by *M. robertsii* using an appressorium along a seta (brown), glandular duct (beige), and trichogen cell (purple) followed by budding off of yeast-like blastospores in the hemolymph. (**C, D, and E**) Shown are *M. robertsii*-infected fly wings incubated with specific histochemical substrates to demonstrate aminopeptidase, subtilisin protease, and esterase activity, respectively, on appressoria and appressorial plates as described by St. Leger et al. (154).

tolerance to environmental stresses and increasing their virulence (21, 22). Up until now, most "useful" genes for strain enhancement have come from pathogens themselves, from host insects, or from arthropods, such as spiders, that produce insect-specific toxins (Fig. 2A) (23–27, 155). Each of these sources provides a vast array of biologically active metabolites. In addition, a *Metarhizium* strain has been engineered that expresses a single-chain antibody fragment that blocks transmission of malaria parasites (27). Recombinant antibodies provide an additional vast array of potential anti-insect effectors that could target, for example, hormone receptors. The efficacy of *Metarhizium* strains expressing arthropod toxins or antimalarials is currently being tested in a purpose-built "Mosquito Sphere" (a compartmentalized facility with double-walled mosquito netting designed to test experimental fungi and insects in a natural ecosystem) in the malaria endemic region of Soumousso, Burkina Faso. The desire to use these new tools safely has driven studies on monitoring and mitigation methodologies for confinement of genetically engineered (GE) *Metarhizium*; development of bioconfinement strategies; survivability profiles and fitness of GE *Metarhizium* in the wild; studies of transgene stability over many generations; and the "evolvability" of transgenic strains if they escape containment (28). A review of future prospects for biocontrol highlights the application of transgenic *Metarhizium* to mosquitoes as providing GE biopesticides with the high profile necessary for widespread acceptance as safe and sustainable alternatives to chemicals (29).

As well as their direct benefit to agriculture and vector control as insect pathogens, *Cordyceps/Ophiocordyceps* spp., in particular, are medicinally valued because they are generally prolific producers of enzymes and diverse secondary metabolites with activities against insects, fungi, bacteria, viruses, and cancer cells (30–34). Enzymes from *Metarhizium* and *Beauveria* spp. are frequently exploited as industrial catalysts (35, 36), even though the responsible genes for these products are rarely identified. The study of infection and immunity in insects has achieved considerable prominence with the appreciation that their host defense mechanisms share many fundamental characteristics with the innate immune system of vertebrates (14). Studies on the highly tractable model organism *Drosophila,* in particular, have led to a detailed understanding of conserved innate immunity networks, such as Toll (37).

The recent discovery (38) that common entomopathogenic fungi, including *B. bassiana* and *Metarhizium* spp., have epiphytic interactions with plants has generated a plethora of promising avenues of research investigating commonalities and distinctions between these two lifestyles (28, 39, 40). The same molecular and genetic tools developed to probe and manipulate these fungi to better kill insects are quickly allowing development of fungi with altered persistence in the environment and that can function as biofertilizers (40–42).

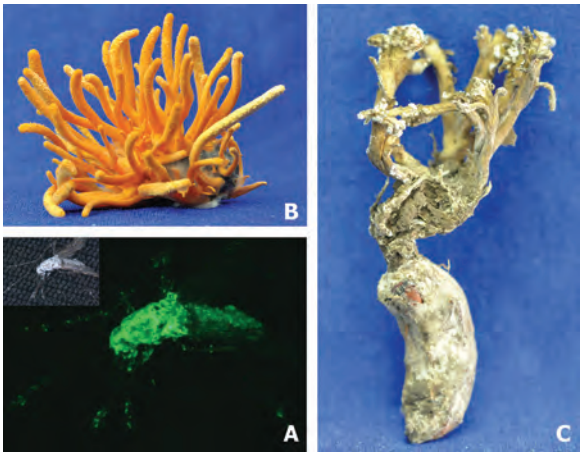


Figure 2 (**A**) Malaria vector mosquito *Anopheles gambiae* killed by a transgenic strain of *Metarhizium* expressing GFP and the spider toxin, Hybrid. (**B**) Fully matured fruiting bodies of *Cordyceps militaris* emerging from a silk worm pupa. (**C**) A fruiting body of *Cordyceps cicadae* forming on the subterranean larvae of its specific host *Cicada flammata*. Images B and C courtesy of Chengshu Wang.

EVOLUTIONARY RELATIONSHIPS OF ENTOMOPATHOGENIC FUNGI

Fungal-insect pathogens represent lifestyle adaptations that have evolved numerous times, and there are significant differences in host range and pathogenic strategies between the major groups (Fig. 3). Ascomycetes, the most speciose phylum, and *Entomophthoromycota* are known to infect a diverse array of insects, Basidiomycota infect mostly *Hemiptera*, and the basal zoosporic groups that produce motile spores that swim to reach their hosts (e.g., aquatic chytrids and blastoclads) usually infect *Diptera* (3). Araújo and Hughes (3) emphasize that, despite their ecological importance and potential applications, fungal-arthropod associations remain an understudied area of fungal biodiversity and likely harbor one of the largest reservoirs of undocumented taxonomic, functional, and genetic diversity within the Fungi.

The best-studied entomopathogenic fungi are the *Entomophthoromycota* and three ascomycete families

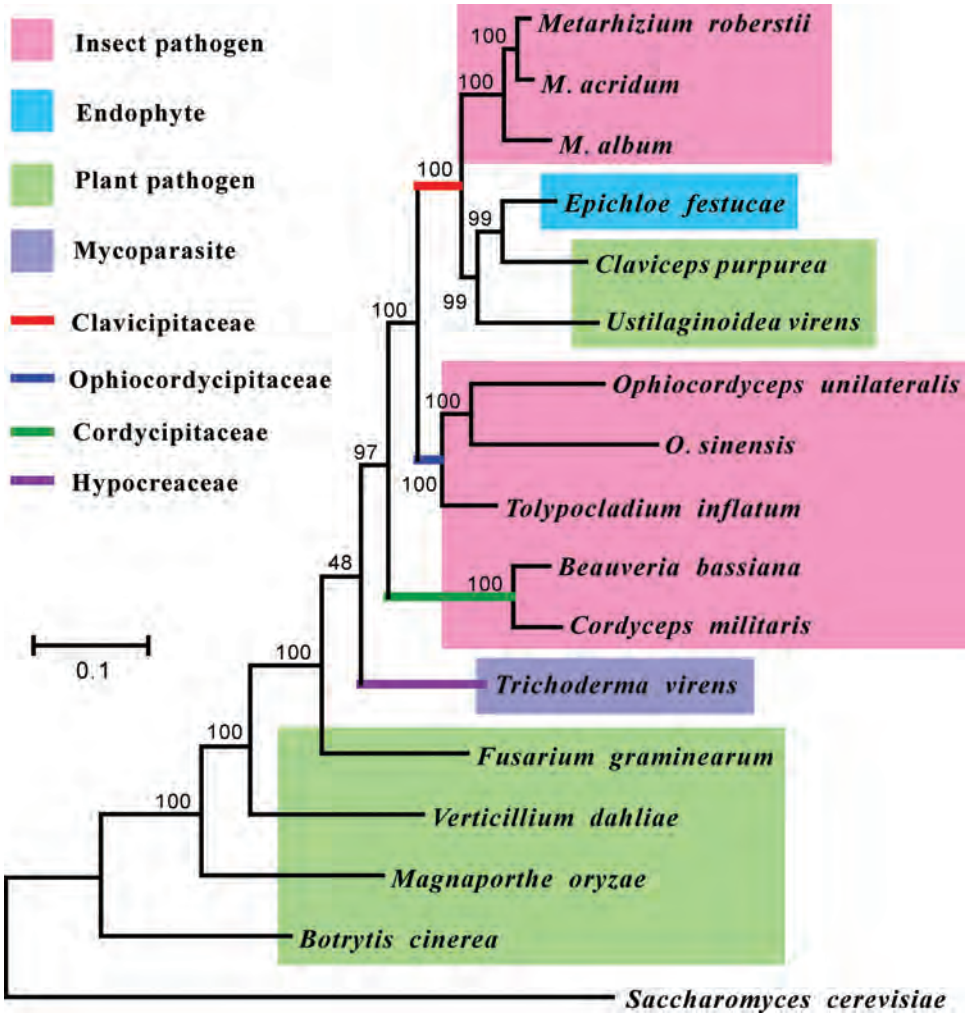


Figure 3 A phylogenetic tree representing relatedness of entomopathogenic fungal taxa. Important entomopathogenic groups are indicated in parentheses. (From reference 15, with permission.)

within the order *Hypocreales* (*Cordycipitaceae*, *Clavicipitaceae*, and *Ophiocordycipitaceae*). In terms of pathogenic strategies, the ascomycetes are characteristically hemibiotrophic, switching from a parasitic, biotrophic phase in the hemocoel to a saprophytic phase colonizing the host cadaver. Conidia are produced on the cadaver, but, unlike the *Entomophthoromycota*, are not actively discharged. In some genera of *Entomophthoromycota*, if the forcibly discharged primary infective spores (ballistospores) miss their aerial targets, they have evolved the ability to form secondary sticky spores (capillispores), thereby creating "minefields" around cadavers to entrap crawling targets (3, 156).

Both *Entomophthoromycota* and *Hypocreales* produce resting structures for persistence in the absence of new hosts or under adverse environmental conditions. The *Hypocreales* have both sexual (teleomorph) and asexual (anamorph) forms, although most research has focused on the anamorphs, which tend to be more amenable to *in vitro* techniques and have greater practical applications. Species in the *Entomophthoromycota* are characteristically biotrophic (exhibiting no somatic growth after death) with a narrow host range and are common among foliar arthropods in temperate environments. Eilenberg and Pell (43) list a number of host-pathogen systems in which the ecology of *Entomophthoromycota* has been studied.

Entomopathogenicity evolved independently in the *Cordycipitaceae*, *Clavicipitaceae*, and *Ophiocordycipitaceae*, and these hypocrealean genera cluster among closely related phytopathogens, endophytes,

and mycoparasites. These ancestral associations are consistent with repeated transitions (host switching) between plant, fungi, and insect hosts (4). Their teleomorphs are the most common fungi encountered in association with arthropods in tropical forests (Fig. 2) (43). Most appear to have a very restricted host range; e.g., the zombie-ant fungus, *Ophiocordyceps unilateralis* species complex (carpenter ant-specific teleomorphs), may have a level of specificity of one fungal species per insect species (Fig. 4) (44–46). In contrast, their anamorphic counterparts are often generalist pathogens with broad host ranges and the ability to pursue both pathogenic and saprophytic lifestyles (47). However, even within anamorph genera, there is considerable variation: thus, *Metarhizium robertsii* can infect hundreds of insect species spanning different orders, while *M. album* and *M. acridum* are specific to hemipterans (true bugs) and acridids (locusts and grasshoppers), respectively (48). In terms of the evolution of fungal host specificity, the study of *Metarhizium* species with different host ranges has shown a directional trajectory of speciation from being specialists to becoming generalists, and the specialists may have cryptic sexual stages (48). As discussed later, this raises the basic question of what is driving the host specificity of the sexual forms to a degree not observed in asexual forms of the same species. Other basic questions that remain unanswered include: why do teleomorphic ascomycetes not occur widely in temperate habitats, and are the teleomorphic ascomycetes utilizing the functional niches in the tropics that *Entomophthoromycota* occupy in temperate zones (49)?

Researchers studying the *Entomophthoromycota*, which are difficult to mass-produce, have focused on the ecology of these organisms and their role as causative agents of mass epizootics. Hajek and Delalibera (50) conclude that they have been used more frequently than other types of pathogens in classical biological control and provide a sustainable avenue for controlling arthropod pests, especially the increasing numbers of invasive species. As of February 2016, 10 strains of entomophthoromycotans have sequencing projects listed on the Genomes OnLine Database (GOLD): three are *Entomophthoraceae*, three are *Basidiobolaceae*, and four are *Ancylistaceae*. Leveraging the full power of genomics in the study of *Entomophthoromycota* will be shortly forthcoming, but currently the only complete genome in this collection is that for a strain of *Conidiobolus coronatus* (all others are draft genomes or incomplete). Studies on anamorphs within the *Ascomycota*, particularly the genera *Beauveria* and *Metarhizium* (family *Cordycipitaceae* and *Clavicipitaceae*, respectively), dominate the entomopathogen literature because they have been developed as

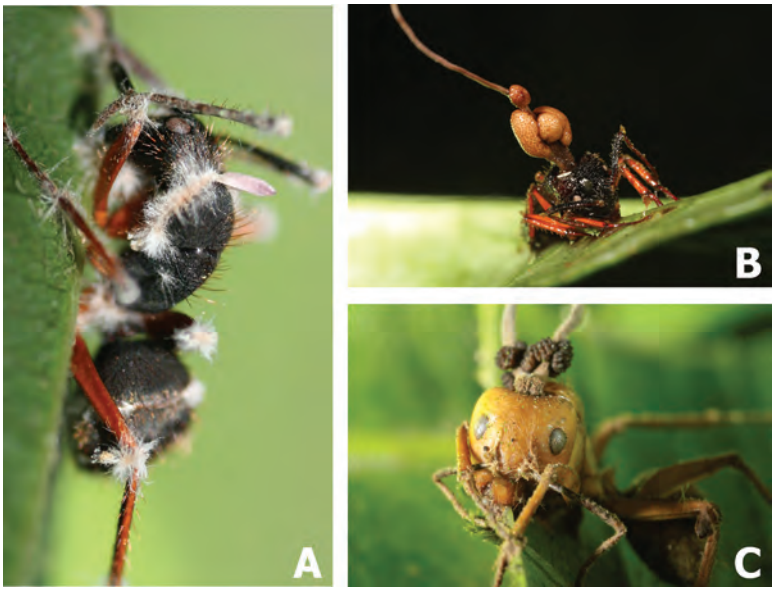


Figure 4 (**A**) A Brazilian carpenter ant *Camponotus rufipes* biting a leaf with *Ophiocordyceps camponoti-rufipedis* just beginning its growth out of the ant's body. (**B**) *Ophiocordyceps unilateralis* fruiting body emerging from the head of the Thai carpenter ant, *Camponotus leonardi*. (**C**) Spore-producing bodies of *Ophiocordyceps camponoti-balzani* on the Brazilian carpenter ant *Camponotus balzani*. Images courtesy of David Hughes.

inundative control agents, which can be applied *en masse* to control a pest population. To date, there is much more genomic information on ascomycete insect pathogens relative to the *Entomophthoromycota* because sequences are available from nine *Metarhizium* strains (48, 51–53), *B. bassiana* (54), *Cordyceps militaris* (32), *Ophiocordyceps sinensis* (anamorph, *Hirsutella sinensis*) (33), *O. unilateralis* (55), *Tolypocladium inflatum* (57), and *Hirsutella thompsonii* (56).

The comparative genome analysis of seven *Metarhizium* genomes (48) confirmed it is a monophyletic lineage that diverged from clavicipitacean plant pathogens and endophytes about 231 million years ago (MYA) and placed the hemipteran-specific *M. album* as basal in the *Metarhizium* clade with an estimated divergence time about 117 MYA (48). It has been suggested that the close physical proximity of the plant-associated ancestor of *M. album* to plant-sap-sucking hemipteran bugs may have facilitated this particular host switch to insects (3, 48).

Several *Metarhizium* spp. retain complex relationships with plants and endophytically colonize plant roots and the rhizosphere (the layer of soil influenced by root metabolism) (38, 39, 57, 58). *B. bassiana* also forms intimate endophytic relationships with a broad range of plant species, although it usually colonizes the aerial parts of the plant (59–62). Field trials showed that a rhizosphere competent, avirulent mutant *M. robertsii* strain survived better in grassland soil than an insect pathogenic mutant unable to adhere to root surfaces, providing experimental evidence for the importance of plant roots in maintaining populations of *M. robertsii* (28). Stress may play an important part in adaptive evolution to soil and root conditions (63). Cell wall and stress response genes evolved at an accelerated rate, increasing the fitness of field isolates, whereas virulence determinants were unaltered (28). *M. robertsii* trades insect-derived nitrogen (64) for plant-derived carbohydrates from its plant host (65). Moonjely et al. (39) suggest that, rather than shifting hosts, many of these fungi broadened their host range to exploit insects as a nitrogen source while maintaining mutualistic plant endosymbiosis. They hypothesize that the coupling of these dual lifestyles was the driving force behind this evolution: as conduits of insect-derived nitrogen, these fungi become an indispensable partner underground. As shown by their antagonism to plant pathogenic fungi (66), ability to survive exposure to lead and other heavy metals (67), and pathogenicity to soil amoebae (68), at least some *Metarhizium* isolates have additional unexpected flexibility in their trophic capabilities.

THE FUNGAL INFECTION CYCLE AND HOST SPECIFICITY

Cumulative evidence suggests that genes involved in the specificity of some entomopathogens to a narrow range of insects could control adhesion to the cuticle surface; exploitation of cuticle surface conditions (nutrients, humidity, specific recognition factors); ability to overcome structural and chemical barriers to penetration; and toxin production (48, 51, 69). Much research has been directed at identifying determinants of specificity and virulence, because a major goal in the development of fungal insect pathogens as biocontrol agents is to be able to control these parameters.

Attachment and Penetration of the Insect Cuticle

Although fungal entomopathogens are highly diverse taxonomically, they all produce infective spores that attach to, germinate, and penetrate the cuticle (or digestive tract) of their host. In most cases, spores landing on the epicuticle (the surface layer of the cuticle) undergo a stereotyped series of changes that include producing appressoria (sticky holdfasts that attach to the cuticle) that in turn produce infection pegs that breach the cuticle using a combination of mechanical pressure and cuticle-degrading enzymes (Fig. 1) (70). The nature of the inductive triggers for production of appressoria varies with the entomopathogen; while the protein and chitin composition of insect procuticle appears similar in all insects, the overlying epicuticular components are extremely heterogeneous, even within the same insect genus, and therefore allowing different pathogen responses to particular insects (70).

The insect epicuticle comprises a heterogeneous mixture of long-chain alkanes, wax esters, and fatty acids, and represents the site of adhesion and the first barrier against fungal attack. Hydrophobic interactions initially drive host adhesion, with *B. bassiana* and *Metarhizium* spp. conidia being covered with a proteinaceous layer formed by hydrophobins that contribute variously to cell surface hydrophobicity and mediate adhesion to the similarly hydrophobic epicuticle (71–75). Following nonspecific adhesion via hydrophobins, a specific adhesin-like protein (MAD1) mediates spore attachment in *M. robertsii* (40), and growing germ tubes and appressoria produce a sticky mucilage that also functions to localize secreted cuticle-degrading enzymes in the vicinity of the fungus (Fig. 1C through E) (78).

The transition from polarized hyphal growth to germ tube expansion (appressoria) in *M. robertsii* is linked to a reduced displacement rate or dispersal of

the Spitzenkörper (the vesicle-generating apparatus at the hyphal tip), redirecting cell wall synthesis from the apical tip to the entire cell surface, and is regulated by several signaling cascades including cAMP and Ca^{2+}-dependent phosphorylation events, with mitogen-activated protein (MAP) kinase regulating the organization of microtubules for vesicle transport (70, 76). The inability of the locust specialist *M. acridum* to infect cicadas is determined at the stage of appressorial formation. *M. acridum* adheres and germinates on cicada cuticle, but only forms few, small appressoria, suggesting different stimuli are on the locust and cicada cuticles. These stimuli are likely to be chemical, rather than physical, because locust cuticle extract is sufficient to stimulate abundant appressorial formation against a flat surface (70). Nutrient levels are one of the interactive phenomena that enable isolates of *Metarhizium* spp. to determine whether they are on an appropriate host cuticle (71, 77). For example, endogenous nutrients on homopterans are supplemented by sugar-rich insect secretions, while available nutrient levels on beetles are very low. Consistent with this, coleopteran-derived isolates produce appressoria against a plastic surface only in the presence of low levels of complex nitrogenous nutrients, while many lines isolated from homopterans also produce appressoria in glucose medium (71). Strains with very narrow host ranges showed the least plasticity and require host-derived supplements to stimulate germination and differentiation (70, 78). Despite their different, presumably selectable, responses to host-related stimuli, the ultimate physiological and morphological responses of these isolates (in terms of appressoria formation, enzyme biosynthesis, etc.) are very similar. This suggests host recognition may be determined by regulatory controls that allow modulated expression of pathogenicity genes by these fungi when they are on a suitable host; conversely, these regulatory controls preclude the expression of pathogenicity genes when not on a suitable host. Current evidence suggests the power to differentiate between hosts depends on G-protein-coupled receptors (GPCRs) that sense extracellular cues. *Beauveria* mutants defective in the GPCR3 gene showed reduced virulence consistent with a role for GPCRs in initial infection stages (79). Compared with the specialists *M. album* and *M. acridum*, there was a major expansion of GPCR-related proteins in the broad-host-range *Metarhizium* spp., consistent with these being able to recognize and respond to many more environmental triggers, with particular expansion in the subfamilies that are developmentally upregulated early in germination and formation of infection structures on host cuticles (48, 51). *M. acridum*, but not *M. robertsii*, transcribed different *Pth11*-like GPCR genes on locust and cockroach cuticles, indicating a role in coordinating a response to specific hosts, or at least that these genes have a function that varies between strains with different host ranges (51).

M. acridum utilizes hydrocarbons and long-chain fatty acid components of the epicuticle during its prepenetration growth, but the epicuticle also contains growth-stimulating peptides, free amino acids, and sugars (80). These induce extensive growth and appressoria formation by *M. acridum*, whereas the fungus is largely unresponsive to the nonpolar lipid fraction (a mixture of long-chain *n*-alkanes) that makes up the bulk of the locust epicuticle (79, 81). Thus, it seems that simple polar compounds are required to stimulate germination before the fungus can make effective use of a complex mixture of nonpolar lipids (80). At high levels, a polar extract of locust epicuticle produced extensive hyphal growth at the expense of appressorial production (81). This is consistent with observations on *M. robertsii* that suggest that a primary role of the appressorium is to establish a nutritional relationship with the host and is not necessary in the presence of a sufficiency of nutrients (82). *M. robertsii* and *B. bassiana* upregulate cytochrome P450 (CYP) subfamily CYP52 enzymes (for metabolism of insect epicuticular lipids) and lipase genes as they germinate on the cuticle surface (51, 54). Conidia harvested from insect cadavers contain high levels of cuticle-degrading enzymes and display higher virulence (83). Growth on insect lipid extracts can also result in conidia displaying higher virulence (84), presumably due to priming of lipid assimilatory pathways (85). However, individual targeted gene knockouts of the eight *B. bassiana* cytochrome P450 enzymes demonstrated a role for only one of these (Cyp52X1) in targeting cuticular waxy layer components (85, 86).

Following initial germination and growth of the fungal germling on the epicuticle, it proceeds to penetrate the protein/chitin insect procuticle. This involves the sequential expression of different degradative enzymes in combination with mechanical pressure. Mechanical pressure in *M. robertsii* appressoria is exerted by breakdown of endogenous lipids to glycerol; this process increases turgor pressure and is mediated by the expression of Mpl1 (perilipin) (87). Genes specifically induced by cuticle included a plethora of cuticle-degrading enzymes and transporters for cuticle degradation products. Although *M. robertsii* has specific responses for different cuticles, the most highly expressed cuticle-degrading enzymes are a large combination of subtilisin proteases

(88). These differ in regulation (88), as well as structure and function (89). Thus, the broad-range subtilisin Pr1A is expressed by appressoria (90) as part of a general response to nutrient deprivation and functions in concert with the exopeptidases to generate host degradation products. These products allow the fungus to "sample" the cuticle and then respond with the secretion of an arsenal of cuticle-induced proteins, including proteases that require cuticle for induction, have different specificities, and have specialized roles in breaching host barriers (88, 89).

Comparative genomics has shown that diverse entomopathogens have more proteases, particularly subtilisins and trypsins, than plant pathogenic fungi (48, 51); this is consistent with niche-specific traits, i.e., traits shared by fungi that occupy the same niche irrespective of their phylogenetic position (91). The expansion of proteases is dramatic in *B. bassiana* and broad-host-range *Metarhizium* species, and less marked in more specialized pathogens, e.g., *M. acridum* and *Cordyceps* species. Similarly, entomopathogens have greatly expanded families of chitinases, lipases, fatty acid hydroxylases, and acyl-CoA dehydrogenases (for β-oxidation of fatty acids), compared with plant pathogens, and the generalists overall have more of these enzymes than the narrow-host-range species (32, 51, 54). The basal *M. album* genome, in particular, highlights the early expansion of genes involved in cuticle degradation, because it has 3-fold or more trypsin genes than related plant endophytes and phytopathogens (Fig. 3) (48). However, compared with *M. album* (87 proteases) and *M. acridum* (116 proteases), there has been additional expansion of proteolytic capacity in other *Metarhizium* species (average 165 proteases). Therefore, the proliferation of proteases may reflect an adaptation to infect insects via the cuticle and, as with the GPCRs, is possibly influenced by host range. The abundance of chitinases in entomopathogens compared with plant pathogens is likely an adaptation to the abundance of chitin in the insect cuticle. For entomopathogens, the necessity of crossing the protein-chitin procuticle has, therefore, had a major impact on their evolution; this is also a testament to the critical role the cuticle plays as a defense against entomopathogenic fungi. Genetically engineering overexpression of chitinase and protease activities has led to the construction of more virulent strains of *Metarhizium* and *Beauveria* spp., suggesting that these enzyme activities may be (partially) limiting virulence in the wild type (92–95).

Clearly, there are factors specialists lack that limit their ability to cause disease in multiple insects, as demonstrated by an increased host range following transfer of genes from a generalist strain to the locust specialist *M. acridum* (96). Interestingly, the components of pathogenicity in specialists and generalists differ not only in how fast they evolve, but also in the ways in which they change. Thus, *M. acridum* has a large number of rapidly evolving genes (those with abundant nonsynonymous mutations), compared with generalists, showing that this specialist has not remained functionally static; rather, specialization has involved rapid evolution of existing protein sequences rather than the extensive gene duplication observed in generalists (48). The rapidly evolved genes are genes involved in specific locust to *M. acridum* pathogen interactions and, by evolving under pairwise coevolution, have been subject to strong balancing or directional selection. By contrast, generalist *Metarhizium* spp. interact with a wide range of hosts in multiple environmental conditions. Diffuse coevolution with many insect hosts offers an explanation as to why signatures of positive selection are observed less frequently in the genomes of *M. robertsii* and other generalists.

With the exception of *O. sinensis,* insect pathogenic ascomycetes have a 2- to 3-fold higher proportion of their genome (~17%) devoted to secreted products than other ascomycetes, including plant pathogens and mycoparasitic *Trichoderma* spp. (54). *O. sinensis*, which mummifies ghost moth larvae (*Thitarodes* spp.) exclusively in Tibetan Plateau alpine ecosystems, provides an extreme case of specialization. Touted as "Himalayan Viagra," the fungus' sexual fruiting body is highly prized because of its pharmaceutical activities and dwindling supply (33). *O. sinensis* infects caterpillars through their spiracles (breathing holes) or orally and has greatly reduced gene families encoding epicuticular degrading CYP52 enzymes, cuticle-degrading proteases, and chitinases (33). In addition, protein families involved in adhesion to cuticles and formation of appressoria are absent or reduced in *O. sinensis* (33). These gene losses are consistent with the inability of *O. sinensis* to breach intact cuticle. Hydrolytic enzymes, particularly proteases, can elicit host immune defenses (92). In which case, the reduced number of cuticle-degrading enzymes in *O. sinensis* might also be an adaptation to avoid immune system detection during the several years it spends latent in the host. Copy number reduction was also evident for genes encoding known pathogen-associated molecular patterns such as lectins, consistent with selection for "stealth" (avoidance of host defenses) as a major force driving *O. sinensis* evolution (33). Evidently, differences found among the insect pathogens in protein

family size are related to their *modus operandi* and host range.

The large secretomes of insect pathogens probably reflect the many habitats they must adapt to *in insecta*, including the cuticle and the hemolymph, as well as additional environmental habitats in the soil and with plants. These complex lifestyles are also reflected in the transcriptomes of insect pathogens; hundreds of different genes are induced during adaptation to host cuticle, hemolymph, or root exudate (54, 65, 70, 88, 97–99). Some of these genes have been knocked out to confirm involvement in virulence. These encode regulators such as adenylate cyclase, the key enzyme for production of cAMP (100), the cAMP-dependent protein kinase A (PKA) that controls expression of many secreted virulence factors (101), calcineurin pathways with roles in *B. bassiana* responses to environmental stresses and host signals (102), components of the MAP kinase pathways involved in fungal adhesion and penetration (103, 104), an osmosensor that signals to penetrant hyphae that they have reached the hemocoel (105), and perilipin, glycerol-3-phosphate acyltransferase, and cell autophagy-related proteins that regulate lipolysis, turgor pressure, and formation of infection structures (51, 87, 106).

Many of the signal transduction genes regulating virulence in *Beauveria* and *Metarhizium* have also been implicated in pathogenicity in plant pathogens, e.g., PKA, MAP kinase pathways, and GPCR genes. Thus, although the signals that induce germination and differentiation are different, similar signal transduction pathways may mediate these signals in plant and insect pathogens. This conservation of developmental circuitry has probably facilitated switching between very different hosts.

Some genes are highly adapted to the specific needs of *Metarhizium*, e.g., *Mcl1* (involved in immune evasion) with its collagen domain is so far unique to *Metarhizium* and is only expressed in the hemolymph (98). Mr-NPC2a is also expressed exclusively in the hemolymph; it was horizontally acquired from an insect and allows *Metarhizium* to compete with the host for growth-limiting sterols in the hemolymph (107). *M. robertsii* upregulates a specific plant adhesin in the presence of plants (Mad2) and a specific insect adhesin (Mad1) in the presence of insect cuticle, demonstrating that it has specialist genes for a bifunctional lifestyle (40). Other specifically regulated genes include a novel oligosaccharide transporter for root-derived nutrients required to colonize the rhizosphere and roots (65), an RNA binding protein that has important roles in both saprotrophy and pathogenicity (108), and an invertase that aids in the regulation of hydrolytic enzymes and provides a plant-derived signal restricting fungal growth (109).

Transcription of these genes is in part controlled by bZIP or C2H2-type transcription factors (TFs) (110, 111). *M. robertsii* has an array of 24 bZIP domain-containing TFs; a knockout of one of these (*MBZ1*) revealed that it contributes to negative regulation of subtilisin proteases, but positive control of adhesin *MAD1* (110), consistent with transcriptomic data showing little subtilisin production while spores are in the process of adhering to cuticle (51). Characterization of MrpacC, a *M. robertsii* homologue of the C2H2-type PacC TF (a pH-responsive transcription factor), showed that it was highly activated in alkaline conditions and impacts cuticle penetration and evasion of the host immune response, perhaps in part because it positively controlled chitinase genes (111). Cultures of *M. robertsii* always show a rapid increase in pH during production of chitinases (112), hydrophobin, and proteases (113, 114). *M. robertsii* alkalinizes a proteinaceous microenvironment, such as the insect cuticle, by producing ammonia from proteolytic degradation products (114). Digestion of cuticle proteins exposes the underlying chitin to enzymolysis (115), so linking alkalinization with chitinase production is adaptive for the fungus. It is likely that continuing the dissection of the roles of individual TFs in the manner of *MBZ1* and *MrPacC* will untangle the complexity and illuminate the interactions between what currently seem to be disconnected strands of biochemical and molecular data.

Immune Evasion and Growth Within the Hemocoel

Once within the host, entomopathogenic fungi proliferate as a progression of single- or multi-celled structures (protoplasts [fungal cells without a cell wall], blastospores [yeast-like budded cells], hyphal bodies [chains of budded cells]) thought to be important for dissemination of the pathogen. These exploit the nutritional resources of their hosts, and ultimately kill them through starvation or as a result of toxin production. In the majority of entomopathogenic fungi, the hyphae break through the cuticle only after death to produce either more infective conidia for immediate transmission or resting structures (sexual or asexual resting spores, chlamydospores, mummified hosts) for persistence in the environment. Species in the *Entomophthoromycota* are obligate pathogens and do not produce toxins of importance for the progression of the infection. They are characteristically biotrophic, keeping the host alive until all resources are utilized. This is in

contrast to the strategy used by the hypocrealean fungi, which are hemibiotrophic, switching from a biotrophic phase (parasitism) in the hemocoel to a saprophytic phase, colonizing the body after death. Host death is, for broad-host-range strains, usually achieved by the production of toxic secondary metabolites.

Once inside the insect's body cavity, a fungal pathogen faces a potent immune defense by which the host attempts to eliminate or reduce an infection. The antifungal immune response in *Drosophila* includes coagulation or melanization, phagocytosis, cellular encapsulation, and the release of antimicrobial peptides (AMPs) following activation of the Toll signal-transduction pathway (14). Utilizing *Metarhizium anisopliae* in a *Drosophila* mutagenesis screen has enabled investigations on how host genotypes differentially affect pathogen fitness, and how defense is interconnected with other aspects of host physiology in complicated trade-offs (116). Approximately 9% of *Drosophila* lines with random single minos element insertions had altered disease resistance and only 13% of these insertions were in genes encoding immune responses (coagulation, phagocytosis, encapsulation, and melanization) (116). The nonimmune genes impacted a wide variety of biological functions including basic cellular processes, nutrition, early development, and, particularly, behavior (116).

There is evidence that entomopathogenic fungi are capable of interacting with the innate immune system, to successfully infect their hosts. This involves immune evasion strategies that interfere with, disrupt, or manipulate immune defenses (14). *Diptera*-infecting members of the genus *Entomophthora* proliferate within the host insect as protoplasts; producing no cell wall avoids detection by a host immune system that is triggered by cell wall epitopes (117). This intricate mechanism may have been a contributing factor for the evolution of a generally narrow host range within this group (118). However, a narrow host range is not a prerequisite for a "camouflage strategy," as generalist *Metarhizium* spp. produce a hemolymph-induced, hydrophilic collagen (Mcl1) coat on cell surfaces to reduce immune detection of hyphal bodies (98). *Metarhizium* conidia can also be internalized and grow within arthropod phagocytic cells where they can avoid additional immune reactions ("anatomical seclusion" strategy), while dispersing through the insect body (119).

The *Drosophila* mutant screen implicated melanization as an effective defense in the fight against entomopathogenic fungi (115). This is consistent with fungal pathogens tightly regulating their own proteases so as not to activate this pathway (88, 120) and with the observation that many entomopathogenic fungi, including *B. bassiana*, have evolved antiphenoloxidase (anti-PO) activity (121) that may, in part, be mediated by secondary metabolites (99). At least some strains of *Metarhizium* and *Beauveria* are immune to drosomycin, the principal *Drosophila* antifungal AMP (116, 122), suggesting that successful adaptation to entomopathogenicity involved evolving multiple overlapping countermeasures to avoid or mitigate the antagonistic effects of responding host immune defenses.

Most hypocrealean insect pathogenic fungi produce large numbers of secondary metabolites (SMs) that are assumed to be part of the ongoing evolutionary arms race between fungi and insects. These compounds are often synthesized by nonribosomal peptide synthetases (NRPSs), polyketide synthetases (PKSs), and terpene cyclases (TCs). Some of the compounds produced by these SM gene clusters have been identified, such as destruxins and serinocyclins in *M. robertsii* (123). However, a multitude of biosynthetic pathways have been uncovered by the newly available *Metarhizium* genome sequences (123). These include pathways likely responsible for known chemistries (e.g., cytochalasins, ovalicin), pathways without candidate products yet known in *Metarhizium*, but similar to those in other fungi (ergot, diketopipearzine, resorcylic acid lactones), and pathways that are so unique that the molecules they produce cannot yet be predicted. These pathways highlight that the capacity of entomopathogenic fungi for secondary metabolite production is far greater than their known chemistry. Accordingly, we have a primitive understanding of how SMs are involved with the interactions of these fungi with other organisms (123).

There is also considerable variability in the number of SM genes among species in the genus *Metarhizium*. In terms of PKS genes and putative polyketides, generalist *Metarhizium* spp., such as *M. robertsii*, possess a greater potential for the production of secondary metabolites than specialist strains and other sequenced ascomycetes (48, 51, 123). Acting collectively with the secretome, the number and diversity of these effectors may contribute to the ability of generalists to infect and kill a much wider variety of insects than specialists. This also relates neatly with pathogenic strategies because *M. robertsii* kills hosts quickly via toxins and grows saprophytically in the cadaver. The destruxins (cyclic peptide toxins) produced by many broad-host-range *Metarhizium* spp. also play a specific role in resisting host immunity, as they have been shown to suppress AMP expression (124) and variously block phagocytosis (125). In contrast, like many specialists, including the *Entomophthoromycota*, *M. acridum*

causes a systemic infection of host tissues before the host dies, which suggests a much smaller role for toxins (54, 126). It seems likely that specialists will have evolved specific adaptations to evade the immune systems of their particular hosts, whereas generalists that lack these specific adaptations produce toxins to kill the host before it has time to mount a significant defense.

Thus far, no *Metarhizium* SM has been shown to be *sine qua non* for pathogenicity (54, 127), except ferricrocin, which is considered by many to not be an SM, as it functions to sequester intracellular iron like many primary metabolites in other organisms (128). This highlights the complexity of virulence and host range in *Metarhizium* spp: the effect of a single toxic SM is not enough to drive either phenomenon (123).

Members of *Clavicipitaceae* (e.g., *Metarhizium* spp.) share few SM pathways with entomopathogens in *Cordycipitaceae* (e.g., *B. bassiana* and *C. militaris*). *Beauveria* produces its own large array of biologically active secondary metabolites (e.g., oosporein, bassianin, tenellin, beauvericin, bassianolides, and beauveriolides) and secreted metabolites putatively involved in pathogenesis and virulence (e.g., oxalic acid) that have potential or realized industrial, pharmaceutical, and agricultural uses (129). As with *Metarhizium* spp., the natural function of most of these metabolites is unknown, but oosporein suppresses insect host immunity (99).

Many other entomopathogens produce SM compounds with pharmaceutical applications and/or roles in antibiosis, pathogenesis, and competitive interactions between organisms (15, 124). For example, *C. militaris* produces cordycepin and cordycepic acids, which have been linked to a variety of benefits from antiaging to sleep-regulating effects (130). *O. sinensis* encodes four terpenoid synthases and one terpenoid cyclase that are absent in other fungi, indicating that it produces novel terpenoids. However, many putative secondary metabolism clusters were conserved between *O. sinensis* and other insect pathogens, providing singular exceptions to the restructuring of the *O. sinensis* genome by repeat elements, and suggesting that physically linking secondary metabolite biosynthetic genes has strong adaptive significance for entomopathogenicity. *Toylypocladium inflatum* produces a range of insecticidal compounds: most notably cyclosporine, an immunosuppressant in humans, as well as in insects, that is exploited to prevent transplant rejection. Phylogenomic analyses revealed complex patterns of homology between the NRPS that encodes for cyclosporin synthetase and those of other secondary metabolites with activities against insects (e.g., beauvericin, destruxins), and demonstrated the roles of module duplication and gene fusion of distantly related NRPS modules in diversification of NRPSs (131).

CONTROL OF INSECT BEHAVIOR BY PARASITIC FUNGI

Entomopathogenic fungi provide many classic examples of pathogens manipulating the behavior of their hosts, the so-called fungal extended phenotype (132–134). In fact, the Fungal Kingdom stands alone in the range, extent, and complexity of their manipulation of arthropod behavior (3). This ability fascinates both scientists and nonscientists alike: it is fascinating because it inspires the macabre and touches on core philosophical issues, such as the existence of free will, and it is of enormous practical importance because parasites are ubiquitous and many have a predilection for the "immunologically privileged" site of the central nervous system. However, this location also provides a parasite with direct "access to the machinery" to alter host behavior, and a very large number take advantage of this. Unfortunately, the significance of such parasitic infections of the brain and "mind" is still underappreciated with limited information on their public health impact, and their overall impact in many areas of ecology, physiology, and evolution. Fungi probably represent a special case in this general field because of several peculiar features that usually include causing host death at the end of an infection cycle. At this stage, the pathogen depends on efficient transmission to maximize its fitness, so selection will favor positioning the dying host in time and space to achieve this. Numerous studies have documented that entomophthoralean fungi kill insect hosts during the late afternoon or evening (135, 136). This ensures that cadavers sporulate under the humid conditions of night to infect new hosts. Rapid transmission is required because the longer a cadaver is present in the field, the greater the chance that it will fall, be overgrown with saprophytic fungi, or be eaten by a scavenger (134). In many cases, the final interactions between a host and pathogen involve the pathogen directing the infected insect to seek an elevated position where wind currents and gravity can effectively disseminate conidia (the so-called "summit disease"). Summit disease has evolved multiple times within both the *Entomophthoromycota* and *Ascomycota*, and often involves an induced death-grip behavior involving the insect's legs and/or jaws with wings outstretched to allow spores to be discharged from the body (132). Carpenter ants (*Camponotus* spp.) are probably the

best known victims (132). An *O. unilateralis* infection transforms these ants into "zombie ants"; ultimately the fungus directs the ant to wander upward and, around noon (possibly after a solar cue), secure itself by biting down on foliage (twigs in temperate woods and leaf margins in rainforests) (Fig. 4) (136, 137). After the death of the host, the fungus sprouts a fruiting body (ascoma) from the back of its head and, after a few weeks of subsequent growth, this starts to rain sexual ascospores down on passing ants as they move on the forest floor or on branches below (132, 138). The *modus operandi* of *Pandora formicae*, an entomophthoromycotan infecting ants in the genus *Formica*, is very similar to the zombie-ant behavior manipulation seen in *O. unilateralis*: *P. formicae* manipulates ants to leave their nest, wander up onto vegetation, and use their mandibles to secure themselves so fungi are well placed for active discharge of conidia from the soft areas of the ant carcass (139, 140).

Very little is known about the mechanisms behind behavioral manipulation, and research has more often ruled out mechanisms than proved them. For example, adult flies killed by entomophthoromycotan fungi are often highly sexually attractive, facilitating spread of infectious propagules, but this is not due to the production of a sex pheromone (141). However, recent transcriptomic studies on ants infected with *Pandora* and *Ophiocordyceps* offer major insights into the mechanisms and extent to which fungi can manipulate their hosts (55, 142). The *in insecta* transcriptome of *P. formicae* elucidated the machinery that articulates its extensive morphological changes in the host, and revealed upregulation of catalases, which protect against host oxidative defenses, along with host-degrading subtilisin- and trypsin-like proteases (142). These proteases were similarly upregulated by *O. unilateralis* in the death-grip phase of infection, along with enzymes affecting oxidation-reduction processes and modulating serotonin and dopamine, thus, likely also modulating behavior (55). *O. unilateralis sensu lato* also secretes a plethora of putative compounds that have an effect on behavior, including, potentially, ergot, enterotoxins, alkaloids, polyketides, and nonribosomal peptides (55). Ergot famously causes serotonergic overstimulation of the central nervous system in humans and livestock (143), and polyketides, nonribosomal peptides, and alkaloids can be neuromodulatory agents (144). It has also been hypothesized that muscle atrophy seen during an *O. unilateralis* infection may be due in part to fungal enterotoxins (138). When incubated with the brains of two host and two nonhost ant species, *O. unilateralis* produced a different chemical cocktail for each ant species, in a manner that suggested it "knows" the brains of its target hosts and reacts accordingly (44). This goes some way to explaining why different *Ophiocordyceps* species seem to infect only certain ants.

Also of particular interest are potential commonalities for behavior manipulators across kingdoms (55). *O. unilateralis* upregulated the secreted enzyme protein-tyrosine phosphatase (PTP) by >110-fold in its ant hosts. In baculoviruses, PTP has been shown to cause the migration of caterpillars upward into plant foliage before death: a convergent viral approximation of the fungal summit disease (145, 146). The activity of PTP in fungal manipulations of ant behavior has not yet been experimentally confirmed.

Summit disease is not the only behavioral manipulation produced by insect pathogens. In possibly the most highly evolved interactions, as they indicate a very high level of adaptation of the pathogen to the host, the fungus keeps the host alive and controls its flight behavior so that the insect becomes an aerial vehicle for spore release. There are many examples reviewed by Roy et al. (134), but the entomophthoromycotan fungal genera *Strongwellsea* and *Massospora* are the best understood. In the case of *Strongwellsea castrans*, the hosts are adult flies (3). The fungus causes a large circular hole to develop on the host's abdomen, and conidia are ejected from the flying insect through this hole. *Massospora cicadina* also initiates sporulation when its cicada host is still alive (Fig. 5) (147, 148). Eventually, the abdomen is entirely consumed,



Figure 5 Live cicada with its abdomen replaced by sporulating *Massospora cicadina*: the insect host disseminates the fungus during this stage of the disease. Image courtesy of Mike Raupp.

leaving just the head and thorax of the living insect. The cicada's ability to fly is retained, increasing dispersion of spores in the environment, especially in the case of infected male cicadas, which attempt to attract and copulate with females and even continue to feed (3, 149).

EVOLUTION OF SEX IN ENTOMOPATHOGENIC FUNGI

Teleomorph hypocrealean states have much narrower host ranges than anamorphs, prompting the interesting question of how closely related fungi could differ so fundamentally in ecology depending on sexual state (49). Comparative genomics of entomopathogenic fungi has confirmed they exhibit diverse reproductive modes that often determine the rates and patterns of genome evolution and are linked as cause or effect with pathogenic strategies (15). The three modes of sexual reproduction in ascomycetous fungi, i.e., heterothallic, homothallic, and pseudohomothallic, are governed by the mating-type (*MAT*) locus (150, 151). The haploid genome of heterothallic species carries only one of the *MAT* loci, and, thus, they require a haploid partner with a compatible *MAT* locus to complete the sexual cycle. Homothallic species (self-fertile) have both loci in their haploid genomes, while the pseudohomothallic species are similarly self-fertile, but they contain two compatible haploid nuclei within their sexual spores (152). Most members of the three hypocrealean entomopathogen families (*Clavicipitaceae*, *Cordycipitaceae*, and *Ophiocordycipitaceae*) are heterothallic and are therefore potentially outcrossing fungi. Signs of sex in supposedly asexual species include footprints of Repeat Induced Point (RIP) mutations, a genome defense mechanism specific to fungi, occurring only during the sexual stages on repeated sequences. The consequences of RIP mutations are that repeated DNA segments, such as those that would result from the transposition of a retrotransposon or the duplication of a gene, are inactivated by mutations. Calculations of RIP indices indicated that RIP mutations occur in narrow-host-range species, *M. album* and *M. acridum*, but not in the broad-host-range species such as *M. robertsii*, which suggests retention of sexuality in specialists, although their sexual stages have not been verified (48). Intriguingly, therefore, asexuality in *Metarhizium* spp. is associated with broad host ranges and sexuality with narrow host ranges, consistent with the narrow host range of known teleomorph states. Unlike *C. militaris*, which is specific to lepidopteran pupae, the closely related *B. bassiana* has a wide host range. *B. bassiana*, like generalist *Metarhizium* spp., lacks the RIP mutations, consistent with the sexual cycle being rare (54). RIP is incompatible with gene duplication events, so its absence is consistent with expanded gene families and more transposable elements (TEs) in the *B. bassiana* and *M. robertsii* genomes, relative to *C. militaris* and *M. acridum*. It is possible, therefore, that losing sexuality, and therefore RIP, was a prerequisite for generalists expanding gene families.

In contrast to the other sequenced insect pathogens, which are all heterothallic, *O. sinensis* contains two compatible MAT loci in the genome and is sexually self-fertile, i.e., homothallic (33). It is likely that inbreeding is an adaptation by *O. sinensis* to its small population size resulting from a very specialized lifestyle and the extreme environmental conditions in its small geographical range. The *O. sinensis* genome size is approximately three times larger (~120 Mb) than other ascomycete insect pathogens, but, whereas they contain more than 9,500 protein-coding genes, *O. sinensis* has only 6,972 genes (33). The RIP mechanism is dysfunctional in *O. sinensis*, which has probably contributed to a massive proliferation of retrotransposable elements, and thus genome size inflation, and the large number of retrotransposed and fragmented pseudogenes in the genome implicates retrotransposition in most of the gene losses in *O. sinensis*. It has been proposed that plant pathogenic fungal lineages with large and flexible genomes are likely to adapt faster during coevolution with hosts (153). The massive proliferation of transposable elements in the inbreeding *O. sinensis,* may provide a trade-off between advantages of increased genetic variation independent of sexual recombination and deletion of genes dispensable for its specialized pathogenic lifestyle. As *O. sinensis* has lost many genes for expanding its host range, future transitions away from its current lifestyle seem unlikely, indicating that, while retrotransposition may facilitate rapid adaptation, it may also contribute to stable host interactions.

CONCLUSION

Nearly all fungal phyla have representatives that have convergently evolved pathogenicity to insects. Identifying commonalities and differences between these fungi is providing a comprehensive picture of the mechanisms they use for pathogenicity, and how new pathogens emerge with different host ranges. Bioinformatics and sequencing technology have already contributed greatly to the study of insect pathogenic fungi, particularly the hard to culture obligates, and promise further

breakthroughs in some of the more intractable aspects of this field, e.g., behavior manipulation and evolution of host specificity. Bolstered by this influx of information, entomopathogenic fungi are emerging as models for understanding how pathogens in general respond to and evolve in changing environments, initiate host invasion, colonize tissues, and counter host immune responses. These pathogens offer us a glimpse into the diverse answers fungi have to the question, how best to kill an insect?

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Made for Each Other: Ascomycete Yeasts and Insects

46

Meredith Blackwell

INTRODUCTION

The classic work of Paul Buchner in the first half of the 20th century established the common occurrence of symbiotic associations between microbes and animals, often a fungus association hidden from view within an animal gut (see reference 1). Interest in the fungi associated with insects continues (2–9), and recently renewed interest comes from the molecular techniques available to study the interactions.

In the mid-1990s I was asked by a prominent ecologist how I would go about undertaking one of the trendy things in field biology at the time: conducting an all-taxon biological inventory of fungi (10). In addition to surfaces of everything, I suggested sampling the internal parts of every plant and animal within the boundary of the survey. As an entomologist, he recognized the enormity of such an inventory, a task probably not worth the effort with the methods available at the time. Soon after that conversation many new fungus-arthropod associates were discovered using the standard techniques of dissection and culturing internal insect parts, especially intestinal tracts, and with the aid of new DNA sampling methods, progress was faster. Often, newly discovered gut fungi were yeast-like in form (11), and new taxa and lineages of yeasts were found associated with insects. Boekhout (12) pointed out that only 6% of the yeast strains in the CBS-KNAW collection had come from insect sources, a percentage that is increasing today.

YEAST: A POLYPHYLETIC GROWTH FORM IDEAL FOR ANIMAL SYMBIOSIS

"Yeast" is a growth form, and yeasts have common morphological traits but do not form a monophyletic group (13–15). A simple description that covers the disparate taxa of fungi known as yeasts is that "yeasts bud" (Fig. 1). Any fungus that is characterized by production of budding cells in asexual reproduction has been called a yeast or yeast-like fungus (Fig. 1, Fig. 2). Alternative forms of asexual reproduction such as conidium formation also may occur in a budding individual. Pseudohyphae are connected buds that do not separate from each other, and the cells may elongate to look like filaments. True hyphae are developmentally different because they are formed by extension and elongation at the filament apex without budding.

Historically, one subphylum of Ascomycota, Saccharomycotina, has been known as "the yeasts." When yeasts in Saccharomycotina reproduce sexually, they lack specialized sex organs, and ascospores (Fig. 3) are produced in somatic cells converted into naked asci, that is, asci not amassed in a common structure, an ascocarp. Some members of Pezizomycotina (e.g., *Symbiotaphrina* spp. and unnamed obligate associates of planthoppers) have yeast growth and have been called yeasts but also yeast-like symbionts (YLSs), to distinguish them from Saccharomycotina yeasts. In addition to existing as budding cells, some fungi are dimorphic with an individual changing between budding and hyphal growth depending on environmental conditions. Changes occur when certain dimorphic fungi assume yeast morphology in association with an insect host (e.g., the mycangia of many ambrosia beetles) but undergo hyphal growth when they are free-living. A similar switch between growth forms is well known in certain animal pathogens (e.g., Onygenales).

Until recently yeasts have been difficult to identify and classify. Because single cells provide few morphological characters, 19th century workers relied on methods used by bacteriologists. The outcome was 2-fold: the availability of many phenotypic characters and the

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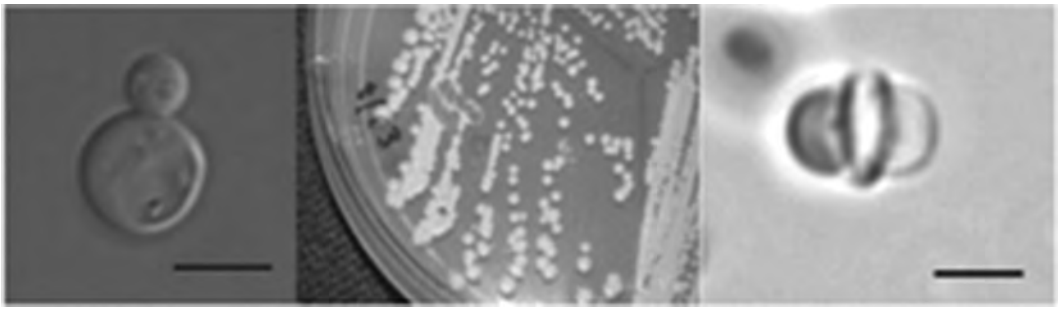


Figure 1 Many yeasts reproduce asexually by budding. *Spathaspora passalidarum* from a culture. Scale bar = 5 μm. Photograph courtesy of N.H. Nguyen.
Figure 2 The edge of a 9m plate showing discrete budding yeast colonies developed on agar after streaking. Photograph courtesy of S.-O. Suh.
Figure 3 Hat-shaped ascospores of *Scheffersomyces stipitis*. Ascospore shape was once thought to be a good phylogenetic character in yeasts. DNA sequencing indicates it has arisen independently several times, not only in yeasts, but also in Pezizomycotina. Scale bar = 5 μm. Photograph courtesy of S.-O. Suh.

realization of the biochemical potential of yeasts. With the onset of DNA-based classification in the 1980s, DNA-DNA reassociation attempted to give a genetic basis for relationships, but methods for yeast identification plus phylogenetic assessment only came later with the development of PCR and the use of the D1/D2 region large subunit ribosomal DNA (rDNA) sequencing methods and a large database for making comparisons (16). A broad sampling of 500 species of ascomycete yeasts and the newly defined species limits acquired by this method were equilibrated with results derived from previous methods, including information on outcrossing. Kurtzman and Robnett (16) found that conspecific strains generally had fewer than 1% nucleotide substitutions in the D1/D2 region, and different species usually were separated by more than 1% substitutions. Beyond a means to separate species, the ability to perform phylogenetic analyses was an equally important outcome for yeast systematics and evolution. The paper marked the beginning of a new era in yeast systematics, and with that start yeast biologists have gone on to sequence many genes and entire genomes (e.g., http://genome.jgi.doe.gov/saccharomycotina/saccharomycotina.info.html).

As mentioned earlier, yeast growth at some stage in the life history is a trait of many fungi that are closely or obligately associated with arthropods, but not all yeasts are documented to be arthropod-associated. The yeasts discussed in this article are ascomycetes, members of Saccharomycotina and Pezizomycotina. The yeast growth form, however, is well established in certain members of other taxonomic groups (Table 1), few of which have insect associations beyond dispersal (e.g., Tremellales, Ustilaginomycotina, Pucciniomycotina, and in addition to the Saccharomycotina among the Ascomycota, the Taphrinomycotina and Pezizomycotina). In the past the assumption was that the yeast form probably arose independently on a number of occasions. Information derived from fungal genomes, however, indicates that not only was the genetic information to determine yeast growth present early in the divergence of the fungal lineage, but a conserved genetic toolkit for the yeast factor persisted in many fungal lineages. The sporadic occurrence of yeast forms by what was described as "latent homology and convergence regulatory evolution" possibly involves parallel diversification of Zn-cluster transcription factors known to regulate switches in yeast-filamentous growth (17). The explanation fits the observed pattern of yeast appearances and disappearances throughout much of the

Table 1 Some common insect-yeast and YLS associations[a]

Phylum: Subphylum	Examples of insect-associated yeasts and YLSs
Ascomycota: Saccharomycotina	Many clades, especially *Suhomyces tanzawaensis*, *Teunomyces kruisii*, cactophilic yeasts, *Ascoidea*
Ascomycota: Pezizomycotina	YLS *Symbiotaphrina* of anobiid beetles, YLS of planthopper, dimorphic ambrosia fungi in Ophiostomataceae and Ceratocystiaceae
Basidiomycota: Pucciniomycotina, Agaricomycotina	*Septobasidium*, sciricid wood wasp associates (*Amylostereum*), ambrosia beetle associates (*Entomocorticium*, *Phlebiopsis* cf. *gigantea*, *Peniophora*, *Flavodon*)

[a]See text for more information. See also references 15, 49, 60, 66, 68, and 70.

fungal tree of life. The triggers of the genetic switches, however, have not been identified.

WHY YEASTS AND INSECTS?

Insects, the most successful group of animals on Earth as measured by their species and individual numbers and the diverse habitats they occupy, lack the ability to synthesize sterols, many vitamins, and enzymes to degrade plant cell wall materials and toxic plant allelochemicals. Many insects depend on microbes, including fungi, for their success. Besides direct ingestion of fungi such as mushrooms, ambrosia fungi (see below), and hyphal gardens in Old World termite and New World ant galleries, many more insects benefit from enzymes used to degrade intractable carbon compounds (18, 19) and toxic plant metabolites. Yeasts provide essential amino acids, vitamins, and sterols and produce allelochemicals to attract the insect dispersers for targeted dispersal to a fresh environment (1). The very word "enzyme," derived from a Greek word meaning "leavened," denotes a process carried out by yeasts. A need for insect "probiotics" to acquire carbon is easy to imagine when one considers the diet of partially rotted wood ingested by some insects throughout their entire lives (see section on passalid beetles below).

Yeasts and other fungi provide sterols and vitamins, and although they do not fix atmospheric nitrogen as prokaryotes do, fungi have enzymes to recycle the nitrogen wastes of insects (see section on planthoppers below). Often, less is known about the symbiotic advantage for the fungus, but it is assumed to be a stable environment with a dependable food source and transportation to a new habitat when the old one is depleted. The general term "symbiosis" is often used to cover all types of interactions (e.g., reference 20). The term "endosymbiont" describes a microbial partner within the body of an insect host; "endocytobiont" indicates that the microbial symbiont is located within cells of the host. The examples discussed in this chapter all involve positive interactions for at least one partner, ranging from "horizontal" dispersal on the surface of insects to obligate associations between yeasts and their hosts, in these cases almost always accompanied by "vertical" transmission of microbes from a parent.

INSECT ADAPTATIONS FOR FUNGAL ASSOCIATIONS

Many arthropods are associated with eubacteria and archaea, but individuals of the same species usually have fungal associates (1). Moran, Dillon, and their colleagues (21–23) discussed the importance of beneficial prokaryotic microorganisms to the success of insects, and many of their ideas apply equally well to fungal associations with insects. Engel and Moran (23) summarized factors involved in initiation, retention, and transfer of insect-associated gut microbes: (i) microbial intake through food, the environment, or social interactions; (ii) appropriate physical conditions (pH, O_2 levels, available nutrients) in the gut; and (iii) microbial transfer by egg smearing, eating of frass (excrement), or feeding by offspring at mouth or anus of mature insects to acquire microbes. Modifications to retain and transmit symbionts are numerous, especially in the insect gut.

Insect intestinal tracts are divided into three chambers: foregut, midgut, and hindgut (Fig. 4); differences in the nutrition of the insects are reflected in the physiology and morphology of the gut regions. The gut varies within an individual over different life stages, especially in insects with "complete" (holometabolous) life cycles (having egg, larva, pupa, and adult stages). Physiological differences of the gut significantly affect microbial symbioses, such as fungi favoring the more acid or aerobic areas. In some cases larvae and adults use different food, and these differences are reflected in modified morphology and microbial communities in a single life cycle. Characteristics associated with symbionts include hindguts that are enlarged and differentiated into several regions as in the hindgut of some wood-ingesting insects (see section on passalid beetles below). In other insects midgut modifications including elaborate gastric caeca are evident (see section on anobiid YLSs below).

Major changes in the gut occur when the chitinous exoskeleton covering the soft bodies of immature insects is lost through molting. The cuticle extends inward, lining the inner surfaces of both foregut and hindgut. As long as the insect continues to grow, the cuticle continues to be shed, and associated foregut and hindgut microbes are lost and must be reacquired. In some cases midgut microbes may be affected when the "peritrophic" membranes lining the midgut are disrupted. Midgut caeca or crypts, however, may help to protect against such loss (23).

Other insect structures that house yeasts are gastric caeca and fat bodies. Gastric caeca are pockets that develop off the anterior midgut. Gastric caeca of roaches, stinkbugs, and some aphids are filled with bacteria, while other insects, especially beetles (e.g., anobiids, erotylids, cerambycids, and tenebrionids), contain yeasts. The fat body is a mass of fatty cells that surround the gut, and this major organ is involved in storage and

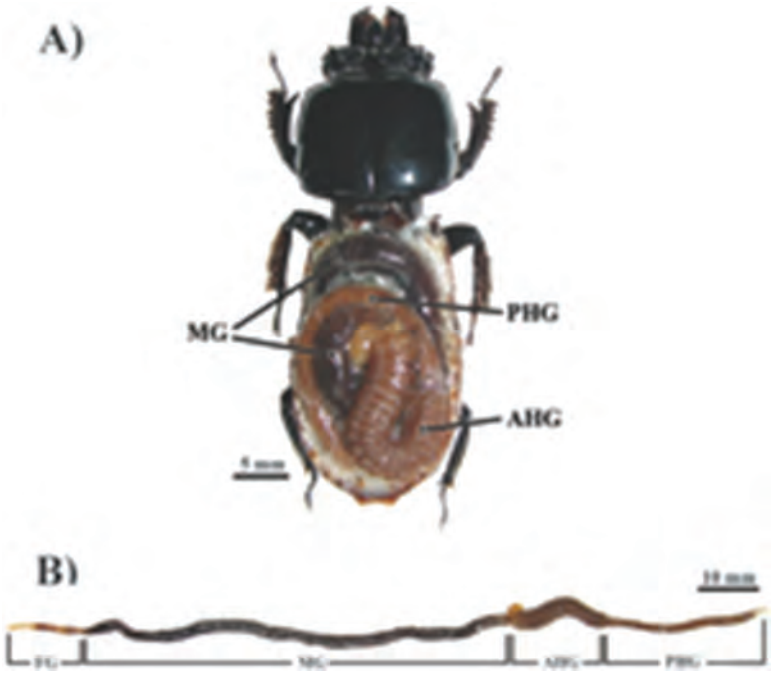


Figure 4 *Odontotaneus disjunctus* (Passalidae). (**A**) Partially dissected beetle shows the gut and its regions *in situ*. (MG, midgut; PHG, posterior hindgut; AHG, anterior hindgut. (**B**) Foregut (FG), MG, AHG, and PHG removed from the beetle. A surface film of attached bacteria is located in the differentiated AHG; *Sheffersomyces stipitis* is attached in the PHG (see Fig. 10) (78). Preparation and photograph courtesy of N.H. Nguyen.

utilization of nutritional reserves, functioning somewhat like a liver. Fat body reserves maintain the insect during energy-depleting activities such as life stage transformations and egg laying. In some planthoppers and aphids the YLSs (see below) are packed into the fat body.

Some insects have invaginations in their exoskeletons that form "mycangia" (pockets) in which they carry fungal spores or yeast cells. Francke-Grosmann (24) discovered "skin glands" in female ambrosia beetles, later renamed "mycangia" (25). The mycangia they studied were glandular structures with secretions probably beneficial to the YLS carried within mycangia. Mycangia can be located on different parts of the insect body, including head, mouthparts, "pronotum" (the covering of all or part of the dorsal thorax) (Fig. 5), sternum, and even legs and wings (26). The use of mycangium has been broadened to include structures that vary in complexity from simple pits and crevices in the exoskeleton to the complex glandular structures that support growth and multiplication of the yeast cells within (27). The term also has been applied to fungus-bearing structures in other beetles (e.g., Lucanidae, Erotylidae), wood wasps (Siricidae), and even the saddlebag-like structures of mites that transport fungal spores in bark beetle habitats.

Insect behavior helps to determine how effective transfer of symbionts to new generations will be. The close contact of "eusocial" insects (those characterized by cooperative broods, overlapping generations, and division of labor into reproductive and nonreproductive types), such as bees, wasps, ants (Hymenoptera), and termites (Isoptera), ensures microbial transmission to the offspring. Other insects, while not considered to

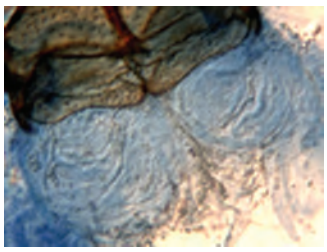


Figure 5 *Xylosandrus amputatus* (Curculionidae: Scolytinae: Xyleborini). Large, complex bilobed mesonotal mycangium (stained blue) with opening on the pronotum; spores of the mycangial symbiont *Ambrosiella nakashimae* at the edge of the pronotum. Fungi in Ceratocystidaceae, including *A. nakashimae*, have close associations with certain species of beetle and produce arthrospore-like cells rather than yeast-like budding typical of other ambrosial symbionts. Photograph courtesy of Chase G. Mayers.



Figure 6 *Pselaphacus nigropunctatus*, a species of beetle (Erotylidae) with parental care; red speckled adult beside a late instar larva (grub) on white basidiocarp of a wood-decaying fungus in Bolivia. Photograph courtesy of J.V. McHugh.

have a high level of social organization, do have parental care, including a surprising number of beetles (Erotylidae [e.g., *Pselaphacus* spp.; Fig. 6], Carabidae, and Curculionidae [Scolytinae, Platypodinae] [J. V. McHugh, personal communication, February 2016]), and many of these insects have microbial associates.

YEASTS IN INSECT HABITATS

Cactophilic Yeast Communities

Drosophila spp. lab stocks are fed yeast diets, so it should not be surprising that the flies are closely associated with yeasts in nature. Experimentation with appropriate yeasts as food for flies continues today (28, 29). In one example genetically modified *Saccharomyces cerevisiae* fed to *Drosophila suzukii*, an invasive pest of soft-skinned fruits such as cherries, may have potential as a RNAi pesticide. Flies fed on the modified yeast cells displayed reduced locomotor activity and other measures of reduced fitness (30).

An understanding of the interactions of cactophilic yeasts, their *Drosophila* dispersers that eat and breed in necrotic lesions of columnar cacti, the cacti, and cactus secondary metabolites comes from more than 40 years of study by William Heed, his students, and colleagues (31–39). The Sonoran Desert assemblages evolved in Arizona and adjacent Mexico under conditions of high stress. Based on their study, Starmer and his colleagues (36) suggested that "cactus chemistry, environmental extremes, and vector associations and interactions" were the likely strong selective factors involved in the origin of the community assemblages. Some of the yeasts detoxify cactus fatty acids and aromatic compounds toxic to the flies, and many of the yeasts have distributions limited by their dispersers (39). A pattern of disperser influence on yeast biogeography also has been seen for yeasts associated with insects in other habitats (e.g., ephemeral flowers and mushrooms; see below). For these reasons yeast populations of columnar cacti, such as senita and saguaro, are relatively stable in species numbers and composition over time and space. In addition to a core group of yeasts associated only with the Sonoran Desert, however, some yeasts are more widespread.

Phylogenetic methods have helped to settle some questions of the origin of the desert yeast communities, which likely are descended from local yeast communities in slime fluxes and fruit rots that evolved as the desert developed. Evidence comes from phylogenies indicating a pattern of many independent yeast origins, rather than diversification from a few common ancestors. Starmer and his colleagues (36) also estimated the age of the cactus-yeast community based on the current ideas of the origin of Cactaceae. Since that time, a more recent date of around 30 to 35 million years ago has been suggested, with current cactus diversity having evolved, perhaps, as recently as 5 to 10 million years ago (40, 41). Additional study of this system offers an opportunity to determine more precisely the rate at which multiple species of yeasts arise under strong selection pressure in desert environments.

Ephemeral Flower Communities

Sap beetles (Nitidulidae) and *Drosophila* spp. are geographically widely distributed, and certain lineages have evolved varied nutritional modes. For example, some nitidulid subfamilies use fermenting plant juices, decaying fruits, or mushrooms as food, while a distinct group visits ephemeral flowers. Lachance and his colleagues (42–47) isolated yeasts from ephemeral flowers (Convolvulaceae, *Hibiscus* spp., Cactaceae) and "insect walk plates" from the flower-visiting beetles in a number of noncontiguous regions, including Hawaii, Australia, Brazil, Costa Rica, Arizona, California, and the Great Lakes region of the United States.

In experiments with bagged morning glory flowers, ascomycete yeast communities did not develop in the flowers, indicating that insect dispersers were the source of yeasts. Many of the yeasts (e.g., *Metschnikowia* spp., *Kodamaea* spp., *Wickerhamiella* spp.) appear to be very specific to the flower habitat (43). The yeast communities isolated from flowers were distinct from those of nonflower tissues of the same plants. The flower-inhabiting yeasts varied in their physiological profiles,

but most had several properties in common (L-sorbose assimilation, lipid hydrolyzation, growth at high osmotic pressures), properties assumed to be correlated with the use of flowers and nectar. In Costa Rica when yeasts from honeybees were compared to those of beetles visiting the same flowers, the insects carried different yeast species, notably *Starmerella* spp., by bees. This study indicated that distinctive communities were determined largely by the dispersers (43, 47).

Mushroom- and Other Fungus-Feeding Beetles

Other yeast-insect associations include beetles that eat mushrooms and other fungi. Certain yeasts were recovered reliably from the gut of the beetles and drosophilids and less often from their habitats (48–50). Drosophilids collected from *Russula* spp. ingested species of *Suhomyces* and *Teunomyces*; the yeasts from the mushroom habitat were distinct when compared to those of other habitats, but the total diversity was low (51). Sexual reproduction is unknown in many of these species, and they appear to be tied to the beetle dispersers rather than the mushroom substrates (48). As mentioned above, cactophilic and ephemeral flower yeast communities also are more closely associated with the dispersers. Little information is available on the interactions between the yeasts and insects, but cultural studies using the standard battery of yeast physiological tests have shown that most of the yeasts produce a wide variety of B group vitamins, ferment glucose and trehalose, and assimilate a wide variety of the carbon sources used to characterize yeasts (52). The common yeast physiological attributes suggest a benefit to the beetles. The presumed absence of sexual reproduction in the yeasts, moreover, implies some degree of dependence of the yeasts on the insect host for transmission, resulting in "mutual beneficiality" discussed by Aanen and Boomsma (53) (see also reference 54).

Nakase isolated *Suhomyces tanzawaensis* in 1966, but he did not formally describe the species until 1988 (as *Candida tanzawaensis*), because he waited in vain to find a second strain. Thirteen years elapsed before any close relatives were discovered (55); eventually the species was recollected and 16 more close relatives were discovered in the habitats and guts of mushroom-feeding beetles (56). Members of the clade, now placed in *Suhomyces* (52), are not rare, only concealed in what was then an unexplored gut niche, especially of erotylid and tenebrionid beetles (Fig. 7). *Suhomyces* spp. have been isolated in the United States, Panama, Japan, The Netherlands, South Africa, and Thailand, almost always in beetle guts or beetle habitats. For example, in a month-long study in Panama, nearly 30% of over 600 yeast strains collected from mushroom-feeding beetles were members of the *S. tanzawaensis* clade (56). The gastric caeca located at the anterior end of erotylid midguts are packed with yeasts, and some of the beetles have parental care, increasing the possibility for transfer of yeasts to the offspring.



Figure 7 The name of the yeast genus *Suhomyces* recognizes Dr. Sung-Oui Suh for his studies of yeasts in the field and laboratory. Barro Colorado Island, Panama, July 2002. Photograph, M. Blackwell.

A trend toward specificity was observed with *Teunomyces* spp. (formerly *Candida kruisii* clade) (52) and mushroom-feeding beetles in Nitidulidae (Fig. 8). *Teunomyces* spp. were isolated not just from nitidulids, but often specifically from *Pallodes* spp. in both Central America and the United States (57). Several other yeast clades are associated with mushroom-feeding beetles, and while there is no strict species-species level specificity, certain patterns have been observed over several geographical regions as was seen in the ephemeral flower communities.

Although filamentous fungi and bacteria may have been present in the gut of the fungus-feeding beetles, none was reported in the studies relying on isolation of microbes on acidified agar medium. Furthermore, usually a single species of yeast was isolated from an individual beetle. A similar trend of low species diversity in drosophilids also was reported (51). Such low microbial gut diversity is unusual, and these observations



Figure 8 Four yeast biologists (l. to r. Drs. Marc-André Lachance, Clete Kurtzman, Jack Fell, and Teun Boekhout) at a coffee break at a meeting to celebrate the centenary of the CBS-KNAW Fungal Biodiversity Centre (since February 10, 2017, known as the Westerdijk Fungal Biology Centre), Utrecht, The Netherlands, in Amsterdam, April 2004. The genus *Teunomyces* recognizes the research of Dr. Boekhout; all the others also have yeast genera named for them and are cited in this chapter. Photograph M. Blackwell.

need to be confirmed. Are they due to artifacts of isolation, the gut physiological conditions, or perhaps the presence of killer strains of *Suhomyces* spp. as previously suggested (S. Lu and M. Blackwell, unpublished observations)?

Ambrosia Beetles Eat Fungi and Their Cultivated Fungal Symbionts

Ambrosia beetles tunnel into sapwood (conducting xylem) to inoculate fungi into the galleries. Some fungi are carried as yeasts in mycangial pouches (Fig. 5), often by female beetles (see above) (24, 25, 58, 59). By the time the eggs have hatched, the yeast-like fungal growth is ready to be larval food. Although described earlier by a forest pathologist, it was the work of Francke-Grosmann that created interest in the ambrosial fungi. Interestingly, Batra's curiosity about ambrosia beetles and their fungi was piqued when he observed ambrosia beetles attracted to beer at a picnic (Lictwardt, personal communication, June 1990).

Many ambrosia fungi are dimorphic, alternating between yeast-like and hyphal phases, growing as yeasts within mycangia and tended in beetle galleries as special hyphae (yeast-like sprout cells described by Batra in 1967) (59) or as more typical hyphae when untended in abandoned galleries. Beetles and fungi form assemblages that evolved independently multiple times, perhaps driven to the xylem to avoid competition in bark habitats (60). Ambrosia beetles are members of the weevil family (Curculionidae), mostly members of Platypodinae, but some also of the derived tribes (Corthylini and Xyleborini) of a second subfamily, Scolytidinae. The fungi, members of the Ophiostomatales, *Ophiostoma* clade (e.g., *Raffaelea* spp.) (61), and the Microascales, *Ceratocystis* clade (*Ambrosiella* and *Meredithiella*) (62, 63), include species associated with both beetle subfamilies. About 3,400 species of Scolytidinae and Platypodinae have an ambrosial habit, but incredibly, only about 35 species of ambrosia fungi (~1%) have been identified from the beetles;

even fewer ambrosia fungi have been given formal names (60).

The taxonomic problems caused by sexual and asexual species related to *Ophiostoma* and *Ceratocystis* are complex, because convergent morphological characters used to promote insect dispersal were previously used in taxonomy. Convergent evolution of many independently arising sexual and asexual taxa and the overwhelming numbers of these fungi require molecular characters for their revision. Great progress has been made when one considers that many of the unrelated fungi once were considered members of a single genus. Although some ambrosia beetles are associated with more than one fungal associate, one species is sometimes designated as the primary symbiont, although this may not always be appropriate. The work of Mayers and his colleagues (63), however, supports specificity of associations between insects with large complex mycangia and their fungal associates, underscoring the probability that many more ambrosia fungi await discovery and description.

An unusual behavior evolved independently on several occasions in some tropical lineages of ambrosia beetles (e.g., *Diuncus* spp.). The beetles lack mycangia to carry their own symbionts, but instead break into the brood chambers of host beetles of a different species to steal their fungal provisions. The behavior could be energetically efficient for the thieves but often is detrimental to the success of the invaded beetles (64). Ambrosia fungi also grow in association with Saccharomycotina yeasts, especially *Ambrosiozyma* spp. (65), and still other beetles are associated with a few basidiomycete yeasts that also are carried in mycangia (66–68).

Most ambrosia beetle-fungal associations do little harm to trees because they provision their galleries in sapwood, but some invasives do cause plant disease (60). The redbay ambrosia beetle, *Xyleborus glabratus*, was introduced relatively recently into the United States from Asia in wooden packing materials (69–71), and the beetle brought with it an aggressive mycangial fungus, *Raffaelea lauricola*, the agent of laurel wilt. Laurel wilt is a lethal vascular wilt disease of southeastern U.S. native redbay (*Persea borbonia*), a member of the laurel family (Lauraceae). Female beetles are attracted to healthy trees by the aromatic volatiles the plants produce, not by aggregation pheromones (or beer) as with other ambrosia beetles. Wilt symptoms occur after the beetle carries the fungus into water-conducting xylem tissues, where the tree reacts by producing "tyloses" (plugging structures) and gums in an attempt to seal off the fungus. Rather, the conducting cells become clogged and water movement upward in the tree is prevented. Symptoms in redbay, such as branch dieback (flagging), followed by rapid death of the tree, are typical of wilt diseases. The beetle and fungus have spread to the south and west, and they have infected additional New World native Lauraceae, including sassafras and several threatened or endangered species, as well as commercial avocado (*Persea americana*) plantings in Florida (69, 70). In the case of a lack of resistance, however, cooperation between fungus and beetle has begun a cascade that may lead to several extinctions in the New World environment. Two species of swallowtail butterflies, the palamedes and spicebush swallowtails, feed largely on three at-risk members of Lauraceae, and butterflies and native laurels are all imperiled (North Carolina Forest Service, http://www.ncforestservice.gov/forest_health/forest_health_laurelwiltfaq.htm).

A pattern of natural selection for resistance in the native region is common in plant and animal infections. For example, Lauraceae in Asia would not be expected to show disease symptoms where the fungus was also native. The same observation can be made in the southeastern United States, where an Asian native of the laurel family, the camphor tree (*Cinnamomum camphora*), was introduced in the late 19th century for commercial purposes that were unsuccessful. The camphor tree has resistance to *R. lauricola* that evolved in Asia and does not have disease symptoms in the New World, but the symptomless plants may act as a reservoir for the fungus (72).

The earliest of all fungus-growing beetles to evolve may have been cucujiform beetles in the family Lymexylidae (73). These beetles have sometimes been called ambrosia beetles, although they are better known as ship-timber beetles. Most species of lymexylids are uncommon, but a few species have caused periodic dramatic losses of living coconut palms as well as of trees and cut timber in southern Asia. In North America certain species have become relatively rare because ships are no longer built of wood, and one beetle has lost most of its specialized habitat to chestnut blight (73).

The ship-timber beetles and their interactions with fungi are poorly known compared to ambrosia beetles in Scolytidinae and Platypodinae. The females of several species are reported to carry yeast cells and eggs in a slimy matrix in vaginal pouches, considered to be mycangia, and to deposit them into weakened or decaying conifer and hardwood trees and building timbers; the movements of early instar larvae have a part in spreading the yeasts to eggs and other larvae in the galleries (58, 74). *Ascoidea africana* (Fig. 9) and *Ascoidea*

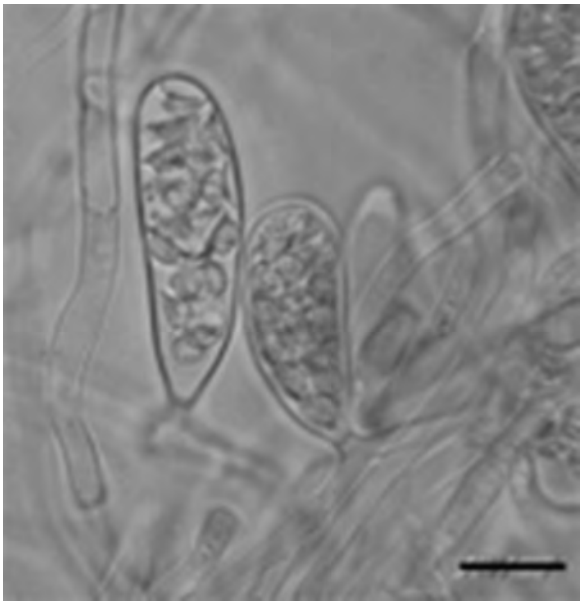


Figure 9 *Ascoidea asiatica* (NRRL Y-17632), an ambrosia fungus disseminated by insect vectors, was isolated from a beetle larva in wood. This species has been reported to be associated with lymexylid ship timber beetles. Asci with hat-shaped ascospores developed on agar, although budding cells and abundant hyphae are also produced under the cultural conditions. Hat-shaped ascospores are a convergent character (see Fig. 3). Scale bar = 10 μm. See reference 133. Photograph courtesy of Cletus Kurtzman.

hylecoeti (Saccharomycotina) have been reported as symbionts of several species of lymexylids, although the yeasts also are found to be free-living (75, 76).

Wood-Ingesting Passalid Beetles

The rich microbial communities of the gut of wood-ingesting beetles, *Odontotaenius disjunctus* and relatives (Passalidae), are separated in compartmentalized guts (Fig. 4) with marked oxygen and pH gradients, sometimes in close proximity (77, 78). Bacteria (e.g., methanogenic and nitrogen-fixing bacteria) as well as yeasts and other eukaryotes (cellulose-degrading flagellates related to those of termites) are partitioned within gut regions that are more anaerobic. Most of the yeasts are able to assimilate xylose (a component of hemicellulose, a plant wall polysaccharide) and some clades ferment xylose (79–81). For example, the xylose-fermenting yeast genus *Scheffersomyces* is attached to the hindgut wall by a holdfast (77), where thalli remain attached in adult and larval beetles as food passes through the gut (Fig. 10). Recently pupated adults reacquire the yeasts after the final molt from gallery walls, provisioned by adults with a mixture of wood and spore-containing frass. Lichtwardt and his colleagues (82) isolated *Scheffersomyces stipitis* (as *Enteroramus dimorphus*) from the hindgut of *O. disjunctus*, where it grew as a filamentous un-yeast-like fungus attached to the beetle gut wall. It was only after DNA sequencing that the fungus was identified as *S. stipitis* (83). In addition to the *Scheffersomyces* clade, members of the *Spathaspora* clade also contain xylose- and cellubiose-fermenting yeasts (81). Because of their ability to ferment xylose to ethanol, biotechnological interest in *Scheffersomyces* spp. and *Spathaspora passalidarum* continues to increase (84, 85). Several other yeasts with similar properties also have been found in rotting wood, and some of these may be efficient for ethanol or xylitol production from sugarcane bagasse (86).

Other yeasts present include additional ascomycete lineages but also several basidiomycete yeast groups, all common in the wood-inhabiting beetle gut in North and Central America, Thailand, and Australia. The yeast lineages usually assimilate xylose, but because many appear to be located in the hindgut, where absorption may be minimal, benefits to the beetle and yeasts are not clear. The yeast communities, however, are fairly stable and present in the gut of all larval and adult wood-feeding beetles that have been examined (80, 87, 88).

Saccharomyces cerevisiae in the Gut of Paper Wasps

In addition to the nutritional and antibiotic benefits of bacteria in the gut of insects, gut conditions in some insects were described as favorable for bacterial conjugation and therefore as "hotspots" for bacterial gene

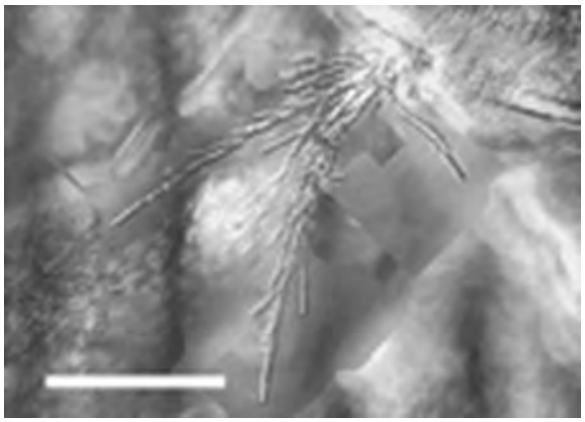


Figure 10 Water mount made without a cover slip of the interior hindgut of *Odontotaenius disjunctus* (Passalidae) showing several thalli attached along a furrow in the hindgut. Scale bar = 100 μm. See reference 77.

transfer (21). A similar phenomenon has been reported for *S. cerevisiae* and *Saccharomyces paradoxus* yeasts in the gut of social wasps (*Polistes* spp.). *S. cerevisiae* is one of the best known of all eukaryotic organisms. As a model organism and the basis of multibillion-dollar-a-year industries, it was the first eukaryotic organism to have its complete genome sequenced. We know, however, much less about its natural history, especially sexual reproduction in nature. On a number of occasions the yeast was isolated from the bark of forest trees, often oak (*Quercus* spp.) (e.g., references 50, 89). Some species of social wasps, also known as "paper wasps," chew wood and bark into a pulp, the paper with which their nest is built, and perhaps chewing on bark or feeding on rotting fruit as they are known to do could be the source of the yeasts in their guts (90) (see also reference 91). Stefanini and her colleagues (90) found interspecific hybrids between *S. cerevisiae* and *S. paradoxus* in the vicinity of their study area, a finding that led to experiments to determine the source of the hybrids. New data supported the presence of high levels of *S. cerevisiae* heterozygosity and interspecific hybrids between *S. cerevisiae* and *S. paradoxus* in the wasp gut. In their experiments Stefanini and her colleagues (92) fed genetically marked strains of the two yeast species to wasps, and hybrids were isolated at increasingly higher levels over time in hibernating wasps. The pure experimental strains of *S. paradoxus* chosen for the experiments could not survive gut passage, but the hybrids did, evidence that in addition to *S. cerevisiae*-*S. cerevisiae* crossing, hybridization with even higher levels of recombination was promoted in the wasp gut.

Anobiid YLSs: *Symbiotaphrina buchneri* and *Symbiotaphrina kochii*

Species of *Symbiotaphrina*, YLSs of beetles in the family Anobiidae, are housed in caeca at the anterior end of the insect midgut, and the female transmits yeast cells to the next generation by smearing them on her eggs. The larvae acquire the cells as they emerge from the eggs. Although the yeasts grow easily in agar culture and have been isolated as endophytes, host beetles have never been found without YLS in nature. Colonies of *S. buchneri* in culture are described as reddish-brown and mucoid, with clavate or pyriform cells having distinctive budding; mycelium is not formed and sexual reproduction is unknown (93).

The yeasts provide nutrients, including B group vitamins and sterols, to their hosts (e.g., reference 94). The yeasts also have attracted interest because the beetles live in processed plant material that contains toxic allelochemicals. *S. kochii* and its host, *Lasioderma serricorne* (cigarette beetle), have survived on tobacco shreds at the bottom of cigarette packs and on other plant materials known to be toxic to insects. Shen and Dowd (95) used the YLS-host system to test the ability of the yeast to survive a variety of plant toxins. The yeasts have broad-spectrum detoxifying enzymes, including ester hydrolase, glucosidase, phosphatase, and glutathione transferase, that they use to detoxify mycotoxins, insecticides, herbicides, and even the organophosphorus insecticide parathion; they used some of the compounds as sole carbon sources.

Escherich (96) first isolated yeast cells of *S. buchneri* from the drugstore beetle, *Stegobium paniceum*; much later, a related yeast, *S. kochii*, was isolated from the cigarette beetle, *L. serricorne* (97, 98). *S. buchneri* was considered a relative of *Saccharomyces* (96), but the placement was questioned because the yeast did not ferment glucose (99). Since that time the yeasts have been considered members of several different genera and higher taxa (see reference 100), but the taxonomic confusion at the higher taxonomic level was settled when small subunit rDNA placed both species within Pezizomycotina but not close to known taxa (100–102). A better understanding of the relationship of *Symbiotaphrina* spp. was to wait for the discovery of a new lineage, the Xylonomycetes. Gazis and her colleagues (103) discovered an endophyte, *Xylona heveae*, in sapwood of Peruvian rubber trees. Surprisingly, *X. heveae* was unrelated to common xylariaceous endophytes, and it lacked the ability to degrade cellulose and lignin, important traits for a plant-invading fungus. More recently, Gazis and her colleagues (104) used additional phylogenomic data to place *Symbiotaphrina* and *Xylona* in a clade within Xylonomycetes, a part of a major radiation of Leotiomyceta within Pezizomycotina. Furthermore, when enzyme profiles and other genome features were compared with those of ascomycetes with varied life styles, *X. heveae* was more similar to animal-associated taxa such as *S. kochii* than to other members of Pezizomycotina, especially those with plant associations (104). Fewer carbohydrate-active enzymes (CAZymes) to degrade plant cell walls were encoded, limiting the ability of *X. heveae* to enter plants by direct penetration. Based on genomic features and the discovery of GenBank sequences of two strains of *Symbiotaphrina* (KC215113, KC215110) that had been isolated as endophytes, Gazis and her colleagues (104) suggested that *X. heveae* could be insect-transmitted. Such a solution to the problem of entry into the plant in the absence of enzymes indicates that the life history of *Symbiotaphrina* may be more complex than previously appreciated.

Planthopper YLSs

Homopterans are sucking insects, including cicadas, planthoppers, leafhoppers, whiteflies, aphids, and scales, many of which transmit plant diseases and all of which harbor intracellular prokaryotes or in a few cases eukaryotic YLSs. A small group of closely related species of YLSs are obligate associates of planthoppers (Delphacidae) and aphids (Aphididae, Cerataphidinae). The fungi are located in "mycetocytes" (fungus-containing cells) in the fat body of planthoppers, and egg surfaces are infected by transovarial infection. The yeast cells are released by exocytosis from fat body cells to the hemocoel, then to cells surrounding the oocytes, and then they finally enter the mature eggs. Larvae become infected as they hatch (1), and the YLSs are present in all life stages of the insects (105). As mentioned earlier, unlike the YLS of anobiid beetles, the planthopper yeasts cannot be cultured on agar; however, a method for obtaining pure YLS cells for DNA extraction involving density gradient centrifugation has supported the research (106).

YLSs are important to the brown planthopper, *Nilaparvata lugens* (Fig. 11), for essential processes, including sterol utilization, nitrogen recycling, and amino acid synthesis (107, 108). Uric acid, an otherwise "waste" product stored by the planthopper, is broken down by the yeast uricase to recycle the nitrogen resource (107, 109–111). The brown planthopper survives on rice phloem sap, which is low in many required nutrients, and this low-quality diet led earlier workers to wonder if the insects might be associated with symbionts. The genomes of four tightly associated organisms (rice, planthopper, YLS, and a newly discovered prokaryotic endosymbiont) revealed the way in which genes of free-living organisms coevolve to the point that some of the organisms can no longer live independently. The reliance on associated organisms includes the eukaryotic YLS, but also the bacterial symbionts present in most of the other groups of Homoptera. The genes of the eukaryotic YLS have functions complementary to those of the planthopper for synthesis of essential amino acids, nitrogen storage and recycling, and sterol synthesis, all of which supplement the poor nutritional quality of rice phloem sap. *De novo* synthesis of essential amino acids occurs in the YLS to supplement the rice sap diet, and some enzymes used in nitrogen metabolism are shared, so that both YLS and planthopper are genetically complementary (112). Fan and colleagues (113) verified previous studies and provided more details of lost genes associated with nutrition and reproduction in the YLS. Strangely, the YLS of *N. lugens*, part of a well-studied model system, has never been validly described, although the name "*Entomomyces delphacidicola*" was suggested (112).



Figure 11 Brown planthopper, *Nilaparvata lugens*, feeding on a rice stem. The insect and YLS harbored in its fat body are obligately associated for essential nutritional resources. Photograph courtesy of Sylvia Villareal, International Rice Research Institute.

Phylogenetic studies indicated that planthopper and aphid YLS are members of the Pezizomycotina (101, 114). Later analyses placed the YLSs within a clade of largely necrotrophic insect pathogens in Ophiostomataceae, including *Ophiostoma sinensis* of medicinal notoriety, and *Elaphocordyceps* spp., parasites of hypogeous mycorrhizal ascomycetes, *Elaphomyces* spp. (115–117). Taxon sampling among Hypocreales has been limited, and only partial small subunit and large subunit rDNA were used in the analyses (115). More information on the position of the YLS among its closest relatives would help to understand the steps involved in the evolution of an obligate symbiotic life style from ancestral necrotrophic parasites.

The genomics of organisms in several symbiotic associations has begun to provide information to support hypotheses of symbiont changes with a change in an insect diet. Current evidence strongly supports the association of a bacterial symbiont similar to *Blattabacterium* in the common ancestor of roaches and termites; soon after the termite lineage diverged, the bacterium was lost in all but the earliest-diverging termite species. The change from prokaryotic to eukaryotic symbionts appears to have been driven by the inability of *Blattabacterium* to synthesize an array of amino acids and to degrade plant cell wall materials during the termite invasion of the woody habitat (118). Replacement of the *Blattabacterium*-like symbiont with hindgut bacteria and protists overcame the dietary deficiencies associated with the ingestion of wood as a sole source of nutrition. Among the homopterans, almost all taxa have bacterial symbionts, but a few lineages have had replacement by eukaryotic YLSs, and replacement of the prokaryotic symbionts by eukaryotic YLSs among several planthopper and aphid lineages probably involved changes in diet as described for the roach-termite symbiont switch. Another outcome of the *N. lugens* genome studies

indicated that a previously undiscovered prokaryotic symbiont was present, indicating the need for caution in the interpretation of symbiotic benefit (112).

ALONG FOR THE RIDE: PHORESY

Botryozyma

Although many yeasts are dispersed by insects, some dispersal (phoretic) associations are highly specialized and involve organisms with interwoven life histories. The term "hyperphoresy" refers to several layers of organisms that all depend on a single mobile organism for transport. Examples include yeasts attached to mites or nematodes, which are attached to the mobile insect. In nature *Botryozyma* spp. are associated with free-living nematodes (*Panagrellus* spp.) (Fig. 12), where they live in insect frass, slime fluxes in trees, and beetle galleries in wood. Kerrigan and Rogers (e.g., references 119, 120) isolated several species of *Botryozyma*, including a sexual state from galleries and frass of the long-horned beetle, *Saperda calcarata* (Cerambycidae), in trembling aspen, *Populus tremuloides*. The yeast has no known detrimental effect on the nematodes or beetles, except to use some energy for transport to fresh habitats. The yeast pseudohyphal filaments attach to nematodes by a bifurcate basal cell and often are present in large numbers.

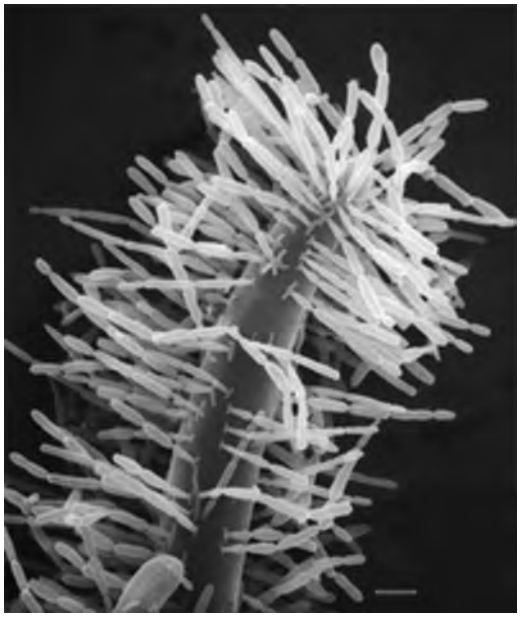


Figure 12 Yeasts are usually studied from isolations made in petri dishes, and their life histories in nature are largely unknown. The yeast described as *Ascobotryozyma americana* (CBS 8560) and the nematode *Panagrellus dubius* have a unique phoretic relationship with the poplar borer beetle (Coleoptera, Cerambycidae, *Saperda calcarata*) that would be unobserved in pure culture. The yeast uses its bifurcate basal cell as a holdfast to attach to the nematode, and they both fly away on a beetle to get to a fresh environment. Scanning electron micrograph, bar = 0.4 mm. See reference 120. Courtesy of J. Kerrigan.

The only other yeast holdfast known anchors *Scheffersomyces pichia* to the hindgut wall of passalid beetles (Fig. 10). The yeasts are not closely related, and the holdfasts are morphologically distinct. Is it possible that other yeasts attach to substrates in nature? We do not know, because yeasts are seldom observed *in situ*, but rather are isolated into culture without direct observation. In addition to *Botryozyma*, a similar dispersal association involves ascospores rather than yeast cells attached to phoretic mites attached to insects (see below).

Yeasts Spread on Substrates

Several other examples of hyperphoresy involve ascospores specialized for insect dispersal. Species in two genera (*Pyxidiophora*, *Kathistes*) of Pezizomycotina are specialized for life in ephemeral substrates such as dung, mushrooms, and rotting wrack strewn on beaches. The timing of maturation is precise, with ascospores, mites, and mobile insect maturing at the same time. The sticky attachment sites of *Pyxidiophora* ascospores become passively attached to phoretic mites, the mites actively seek out and attach to insects, the insects fly to a new substrate, and only after the ascospore reaches the new substrate does a yeast state develop. In cultural studies, ascospores placed on fresh agar produce yeast cells to multiply the number of propagules that then germinate by germ tubes (Fig. 13). The rapid movement of mites across a fresh substrate spreads the yeast cells, probably increasing the chance that the cells will contact hyphae of a suitable fungal host in the complex life history of *Pyxidiophora* spp. (121). In the case of *Pyxidiophora kimbroughii*, perithecia develop in the wood of pine trees, and attachment to a mite disperser is essential to ferry the mites to the bark beetles that undergo their last molt in the outer bark of trees (122, 123).

The ascospores of *Kathistes* spp. also stick to mites, but unlike *Pyxidophora*, they lack a highly specialized attachment region (124). The mites are phoretic on a blood-sucking muscid fly (*Haematobosca alcis*) that blankets the rump of the moose in large numbers (125). The flies spend most of their lives attached to the moose, where both males and females use the blood as their only food. The flies, cued by gasses released when a moose defecates, drop into the moose dung, where

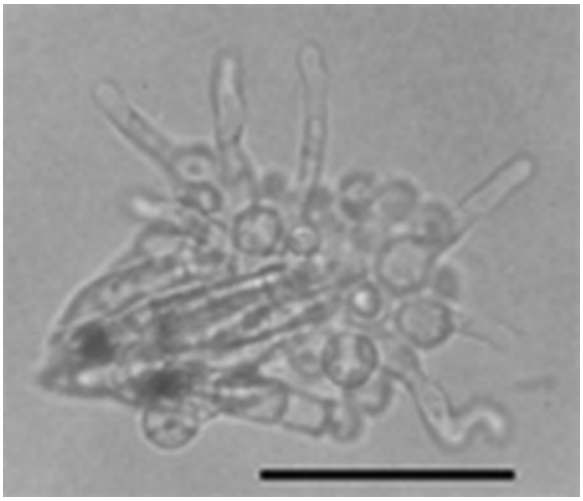


Figure 13 Ascospores of *Pyxidiophora* sp. photographed 24 h after being placed on agar. No cover slip was used, so it looks a little foggy. The ascospore produced several conidia at the ascospore tip, and these budded several times more to produce yeast cells before hyphae elongate. The yeasts are likely dispersed over the new substrate by the mite, improving the chance of finding a suitable fungal host in this complex life history. Scale bar = 50 μm. M. Blackwell.

they lay eggs. *Kathistes*-spore-laden mites hop on flies to return to the moose, and then to another dung pile to repeat the life cycle. The ascospores, aseptate at first, become up to six-septate and germinate by yeast-like budding. In agar culture they usually grow as yeasts. A variety of unrelated ascomycetes have spores that bud in a similar manner, all probably serving the purpose of multiplication of the meiotic products by yeast-like propagules.

EVOLUTION OF YEAST-INSECT ASSOCIATIONS

Because insects and fungi have lived together in terrestrial environments for millions of years (126, 127), it is not surprising that close associations have evolved. Thus far, fossils that could help to clarify steps in the evolution of yeast-insect symbioses have not been discovered. If fossil organisms known to harbor yeasts were found, however, they could provide useful calibration points as they have been used in previous molecular studies (126, 128).

The principle that accelerated evolution is a consequence of the "transition to mutualism" was established soon after the application of molecular methods to the study of fungal interactions (129). These transitions must be millions of years old, providing ample time for associations to develop and communities to assemble, including the polyphyletic assemblages of fungi and ambrosia beetles and cacti, cactophilic yeasts, and *Drosophila* spp., described above. Vega and Dowd (130) suggested three hypotheses of the origin of insect-yeast associations: (i) symbionts were derived from parasites or pathogens; (ii) symbionts were the descendants of plant pathogens or saprobes; or (iii) the insect feeding habitats led to development of the associations. More than one hypothesis may be needed to cover all examples.

Close contact in nature was suggested as the basis for the dramatic host shifts described as interkingdom jumps. In these cases the "host-habitat hypothesis" put forward to explain shifts from one host to another distantly related one states that opportunity for the host switch occurs when both hosts occupy the same immediate environment and rely on the same food source. Examples supported by ancestral character state reconstruction in phylogenetic analyses include host shifts by the YLS member of the Ophiocordycipitaceae jumping from underground cicada larval (insect) hosts to truffle (fungal) hosts and by several shifts in Hypocreales from animals to plants and plants to fungi (131, 132).

CONCLUSION

In this article we have seen examples of progress in the study of symbiosis: investigation of all members of an assemblage and their behavior *in situ* and over time; broad taxon sampling in phylogenetic analyses to pinpoint the closest relatives, then blanketing analyses with near relatives (a process that may rely on long-term searches for undiscovered relatives); examination of fossils by mycologists; and the use of molecular methods for environmental sampling and identification. Lastly, the impact of comparative genomics on our understanding of the evolution of strict associations between yeasts and insects enlightens us, and although many more explanations are needed, the progress shown thus far is encouraging.

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Nematode-Trapping Fungi

47

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INTRODUCTION

There are about 700 species of taxonomically diverse fungi that are be able to attack living nematodes (juveniles, adults, and eggs) and use them as a nutrient source (1, 2). The most important genera include *Purpureocillium*, *Pochonia*, *Hirsutella*, *Nematophthora*, *Arthrobotrys*, *Drechmeria*, *Fusarium*, and *Dactylellina* (3, 4). Among these nematophagous fungi, only a few species are obligate parasites of nematodes, but the majority are facultative saprophytes (5–7). Based on the mechanisms by which they attack nematodes, these nematophagous fungi are usually divided into four general groups: (i) nematode-trapping fungi that use specialized trapping structures differentiated from hyphae; (ii) endoparasitic fungi that use their spores; (iii) the opportunistic fungi that invade or colonize nematode eggs, females, or cysts with their hyphal tips; and (iv) toxin-producing fungi that immobilize nematodes before invasion (1, 8). Nematophagous fungi are important natural enemies of nematodes in soil ecosystems. In recent decades, environmental and health concerns over the use of chemical nematicides have greatly increased the demand for the development of biological control agents in plant protection. The reason for the growing interest in nematophagous fungi is mostly their potential as biocontrol agents against plant- and animal-parasitic nematodes. So far, a substantial number of myconematocides have been developed worldwide (3, 4, 6).

Nematode-trapping fungi are soilborne fungi that entrap free-living nematodes by using trapping structures. These fungi have evolved sophisticated trapping structures, including constricting rings and five types of adhesive traps (sessile adhesive knobs, stalked adhesive knobs, adhesive nets, adhesive columns, and nonconstricting rings) (9–12). About 380 species of nematode-trapping fungi have been reported from different regions of the world (2). Different fungal species can produce one or more types of different trapping devices (11, 12). Most nematode-trapping fungi can live both saprophytically on organic matter and as predators by capturing tiny animals (7, 13–16). Traps are not only the weapons that nematode-trapping fungi use to attack nematodes, but are also an important indicator of their switch from a saprophytic to a predacious lifestyle (10, 16). Nematode-trapping fungi are usually not host specific and can trap all soil-dwelling nematodes (1, 17, 18). With increasing discovery of the corresponding sexual-asexual morph connections, most nematode-trapping fungi are known as asexual morphs of *Orbilia* spp. within the Orbiliales (Ascomycota) (2, 19, 20). Recent analyses based on DNA sequences suggest that trapping devices provide the most relevant morphological features for taxonomic classification of predatory asexual Orbiliaceae (11, 21–23). These fungi have been classified into four genera: *Arthrobotrys* (adhesive three-dimensional networks), *Dactylellina* (stalked adhesive knobs and/or nonconstricting rings), *Drechslerella* (constricting rings), and *Gamsylella* (adhesive branches and unstalked knobs). However, given the new nomenclatural regulations that employ only one name per fungus, the names for these nematode-trapping fungi have to be revised (24, 25).

Previous studies of nematode-trapping fungi mainly focused on investigation of their species diversity and abundance in different regions and habitats, taxonomy and phylogeny, laboratory screening, and field trials for nematode control (4, 6, 15, 26–31). In recent years, more attention has been paid to the molecular mechanisms underlying the process of fungi trapping and infecting nematodes, the origin and evolution of trapping structures, genomics, and proteomics (10, 11, 32–37). Virulent factors, the role of proteases, chitinases and small chemical molecules, and the regulation of trap formation have been extensively studied with modern techniques (16, 37–40). There is an increasing body of evidence to illustrate that the predatory

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lifestyle of nematode-trapping fungi has evolved among cellulolytic or lignolytic fungi as a response to nutrient deficiencies in nitrogen-limiting habitats. Based on molecular clock and fossil evidence, nematode-predatory Orbiliaceae were estimated to have originated as a result of mass extinctions in the Permian and Triassic (10). Recent studies also showed that nematophagous fungi can detect and respond to ascarosides, which are an evolutionarily highly conserved family of small molecules secreted by many species of soil-dwelling nematodes as molecular signal to recognize prey and trigger trap formation (37). By using comparative genomics and transcriptomics, scientists deciphered two genomic mechanisms leading to the evolution of parasitism in nematode-trapping fungi: the formation of novel genes through gene duplication and the differential regulation of existing genes identified by differential expression of orthologous genes (34, 35). Understanding the molecular and genomic basis of nematode-trapping fungi and nematode interactions provides crucial insights for developing effective biological control methods against plant-parasitic nematodes.

TAXONOMY AND DIVERSITY

Nematode-trapping fungi are a taxonomically heterogeneous group of organisms (distributed in Ascomycota, Basidiomycota, and Zygomycota) that can use these special structures (traps) to capture free-living nematodes in soil (1, 17, 41, 42). About 380 species of nematode-trapping fungi have been reported from different regions of the world (2), including species in the genera *Arthrobotrys*, *Cystopage*, *Dactylellina*, *Dactylella*, *Drechslerella*, *Hohenbuehelia*, *Hyphoderma*, *Monacrosporium*, *Nematoctonus*, *Orbilia*, *Stylopage*, *Tridentaria*, *Tripospora*, and *Zoophagus*. Orbiliaceous members represent the largest group of nematode-trapping fungi, which include about 96 species and are currently assigned to the asexual genera *Arthrobotrys* (53 species), *Dactylellina* (28 species), and *Drechslerella* (14 species) (17).

Molecular phylogenetic analyses indicate that the monophyletic classes Orbiliomycetes and Pezizomycetes are among the earliest diverging branches of Pezizomycotina, the largest subphylum of the Ascomycota (43–45). Orbiliomycetes is represented by a single order, Orbiliales, with the only family, Orbiliaceae, consisting of three accepted sexual genera, *Hyalorbilia*, *Orbilia*, and *Pseudorbilia* (46, 47). This family has about 288 described species (19). The members of Orbiliaceae are characterized by small, translucent, waxy, disk-shaped apothecia that are formed in soil and wood. Apothecia are produced without stromata and have an ectal excipulum of globose, angular, or prismatic cells. Small asci with truncate to hemispherical apices are intermixed with paraphyses that typically are swollen at their tips. Small hyaline ascospores usually possess an apical spore body that selectively stains in cresyl blue (48). About 10 asexual genera have been connected with Orbiliaceae (49), and all are hyphomycetous. Certain of these asexual genera are commonly known nematode-trapping fungi, and their evolution and phylogeny have been studied with nuclear and protein-coding genes.

The first nematode-trapping fungus, *Arthrobotrys superba* as the type species of *Arthrobotrys*, was described in 1839, but its nematode-trapping habit was unknown until the observation of *Arthrobotrys oligospora* by Zopf in 1888; the connection between *A. oligospora* and *Orbilia fimicola*, and its nematode-trapping lifestyle, was confirmed by Pfister in 1994, 155 years after the first nematode-trapping fungus was described (50). The characteristics of the genus *Arthrobotrys* were considered to be hyaline conidiophores producing conidia asynchronously on short denticles at swollen conidiogenous heads or clusters of pronounced denticles, the conidia being subhyaline, obovoid, or clavate and (0–)1(–6)-septate (50). Trapping devices were considered to be constricting rings, adhesive nets, hyphae, or adhesive knobs (50). This wide generic concept based on reproductive structures resulted in considerable confusion, although since 1930, nematode-trapping fungi have been mostly assigned to *Arthrobotrys*, *Dactylaria*, *Dactylella*, and some other genera.

The trapping devices of nematode-trapping fungi are developed from the vegetative mycelia. The known trap types include adhesive networks, adhesive knobs, constricting rings, nonconstricting rings, adhesive branches, undifferentiated or unmodified adhesive hyphae, stephanocysts, spiny balls, and acanthocytes (51–54) (Fig. 1). Members of the Orbiliaceae can produce the first five types of traps (9). *Zoophagus* species of Zygomycetes (55, 56) and *Nematoctonus* species of Basidiomycetes can also produce adhesive knobs (57). Traps of adhesive hyphae are restricted to the genera *Stylopage* and *Cystopage* of Zygomycetes (58), while stephanocysts are restricted to the genus *Hyphoderma* of Basidiomycota (51). Spiny balls and acanthocytes are only formed, respectively, by *Coprinus comatus* (53, 54) and *Stropharia rugosoannulata* (59) in Agaricales of Basidiomycota. The sexual morphs of most nematode-trapping species are now assigned to *Orbilia*, and their type of trapping apparatus can be used as robust indicators of generic delimitation. These fungi were

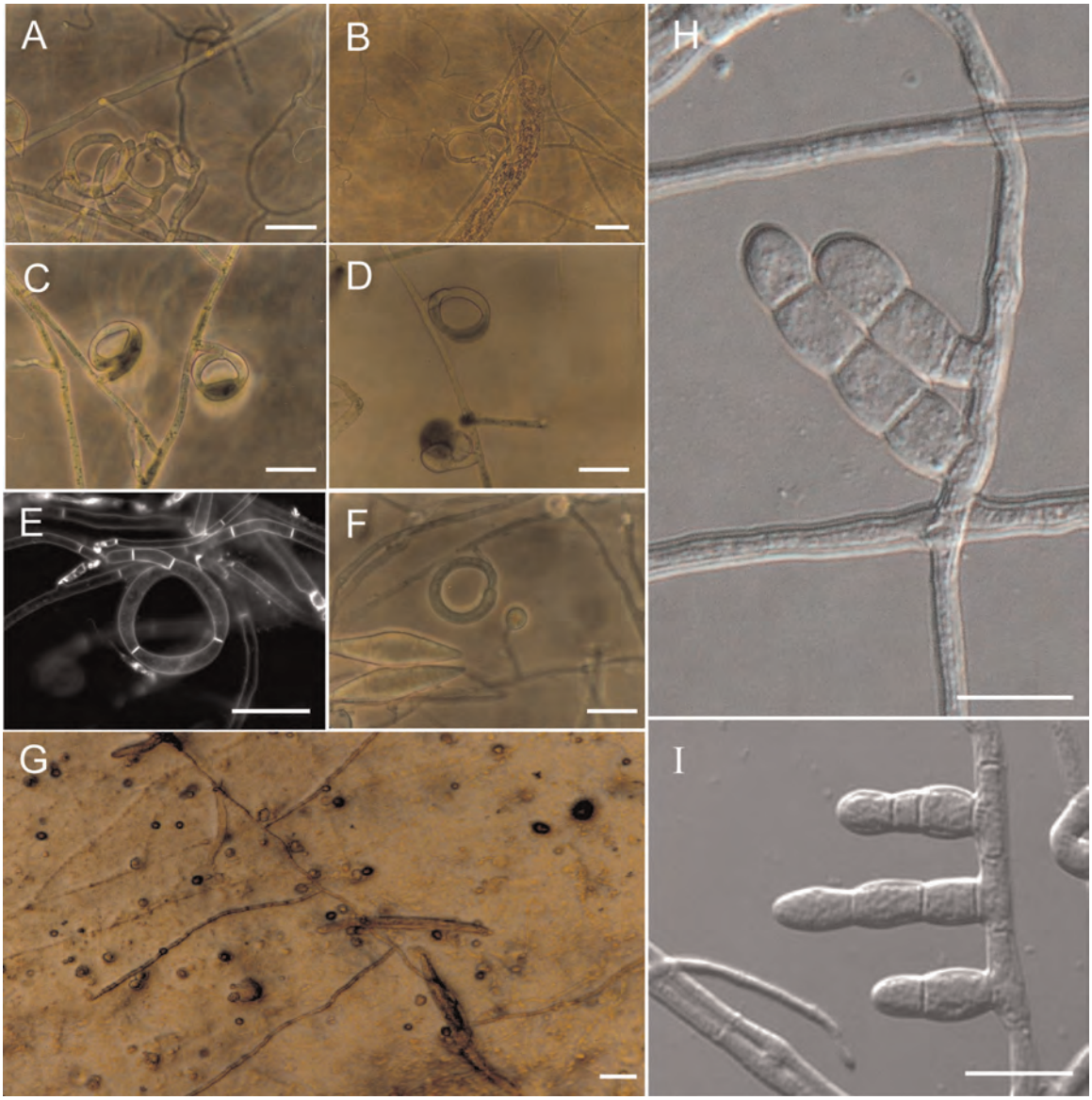


Figure 1 Structures of traps in nematode-trapping fungi. (**A, B**) Adhesive networks of *Arthrobotrys oligospora*. Bar, 20 μm. (**C–E**) Constricting rings of *Drechslerella stenobrocha*. Bar, 20 μm. (**F**) Adhesive knobs and nonconstricting rings of *Dactylellina haptotyla*. Bar, 20 μm. (**G**) Nematode trapped by *D. haptotyla*. Bar, 40 μm. (**H, I**) Adhesive columns of *Gamsylella cionopaga*. Bar, 20 μm.

also classified according to their trap types with the support of genetic data as follows: *Arthrobotrys* (adhesive three-dimensional networks), *Dactylellina* (stalked adhesive knobs and/or nonconstricting rings), *Drechslerella* (constricting rings), and *Gamsylella* (adhesive branches and unstalked knobs) (17).

Fungal taxonomy has changed a lot in the past decades, from being based on only morphological observations to being a combined analysis of morphology and DNA sequence data. Morphology-based classification based on conidia was demonstrated to be inadequate in reflecting natural relationships among the nematode-trapping fungi. The first phylogenetic analysis of nematode-trapping fungi was conducted by Rubner in 1996. She used molecular data to rationalize the classification of nematode-trapping fungi based on types of rapping devices (60). Phylogenetic studies based on rDNA sequence analysis also found that trapping

devices are more informative than morphological characters in delimiting genera (11, 21–23, 61). Subsequently, in 1999 Scholler et al. classified nematode-trapping fungi into four genera using 18S and internal transcribed spacer (ITS) rDNA analysis. Li et al. (21) reevaluated the placement of nematode-trapping genera based on 28S, 5.8S, and β-tubulin analysis, and the establishment of *Gamsylella* proposed by Scholler et al. was criticized and rejected.

Although trapping devices have been shown to be vital in generic delimitation of nematode-trapping fungi, species in the same genus are mainly delimited from each other by the morphology of their conidia and conidiophores. With more and more connections between nematode-trapping fungi and Orbiliaceae being established (49, 50, 62–64), phylogenetic analysis including existing asexual species indicated that conidial morphology showed little correlation, while characters of their sexual morphs showed a high level of correlation. For example, *Arthrobotrys* belongs to series Auricolores, which is defined by the ability to form adhesive networks, while the asexual Trinacrium type belongs to species of *Hyalorbilia* and *Orbilia* subgenus *Hemiorbilia* and subgenus *Orbilia* (H.-O. Baral, personal communication). Further studies are needed to set up a natural classification system based on the connection of asexual and sexual nematode-trapping fungi. In 2012, the International Code of Nomenclature for algae, fungi, and plants was published, which no longer allows a single fungal species to have two names; the older name takes priority except in exceptional circumstances (24, 25). The asexual genus above that is linked to *Orbilia* sexual morphs may need to be allocated to *Orbilia* or, alternatively, to an asexual genus. Discussions are presently under way in the Orbiliomycetes working group of the International Commission on the Taxonomy of Fungi to adopt a single nomenclature for this group of fungi.

ECOLOGY AND EVOLUTION

Nematophagous fungi represent an important group of soil microorganisms that can suppress populations of plant and animal parasitic nematodes (3, 4, 26–28, 65–67). These fungi are broadly distributed in terrestrial and aquatic ecosystems, from the tropics to Antarctica (7, 13, 15, 29–31, 68–70). Efforts have frequently been made to control diseases caused by plant-parasitic nematodes by using nematode-trapping fungi, but results have been inconsistent. It has subsequently been pointed out that a fundamental knowledge of the ecology of these fungi is essential before the value of such biological control methods can be assessed. Environmental factors greatly affect the occurrence and activity of soil microorganisms. However, our knowledge about how the environment affects the abundance and activity of nematophagous fungi is still relatively poor (14, 31).

The effect of major biotic and abiotic variables such as soil moisture, organic matter, pH, nematode density, soil nutrients, and submerged water conditions on the distribution of nematode-trapping fungi has been extensively studied. Previous studies by Gray on the distribution of nematophagous fungi indicate that the occurrence of certain species and groups of fungi is associated with specific soil variables: in particular, pH, soil moisture, nutrients (N, P, K), heavy metal, and nematode density (15, 71). Gray revealed that soil nutrients such as N, P, and K were all positively correlated with nematode density. Based on his results, species with stalked, knobbed trapping devices (*Dactylellina*) and species with constricting rings (*Drechslerella*) were more frequently isolated from organically richer soils which contained a greater density of nematodes (31, 72, 73). However, net-forming species (*Arthrobotrys*) are largely independent of soil fertility, especially low K (74). In a recent survey, Mo et al. found that the diversity of nematode-trapping fungi was positively correlated with lead concentration (75). These soil variables are known to vary with depth, as are the densities of soil bacteria, fungi, and nematodes (76, 77), and a high level of nematode-capturing activity has been recorded from the rhizosphere area. However, there are also large variations depending on plant species and soil types (78). The species of nematode-trapping fungi vary with soil depth (79). Peterson and Katznelson (80) revealed that the greatest diversity occurred in the upper 10 to 30 cm of soils, and this was a positive correlation between the population density of nematophagous fungi and root-knot nematodes in peanut fields. Hao et al. (13) observed that the nematode-trapping fungi were not detected deeper than 4 m in a freshwater pond. Several studies have also been carried out on horizontal distribution (81). For example, Persson et al. (81) studied the growth and dispersion of *A. superba* under natural conditions determined by a radioactive tracing technique.

The number of nematode-trapping species present in a specific soil and their population densities can vary considerably. Application of chopped organic amendment and glucose to soil could increase the activity of nematode-trapping species perhaps because of the consequent increase in the number of free-living and microbivore nematodes (82–84). Although organic

amendments probably stimulated population densities, similar population densities of trapping fungi were found in plots with and without organic amendments. The effect of abscisic acid and nitric oxide on the nematode-trapping fungus *Drechslerella stenobrocha* AS6.1 was tested, and it was demonstrated that the trap development and nematode-trapping capability of *D. stenobrocha* were increased by abscisic acid but decreased by nitric oxide (85). It is apparent that the trapping fungi need a carbohydrate source for their proliferation, but other factors, such as those that cause fungistasis, are also important for their abundance and trophic state in soil. It is hypothesized that *Orbilia* species, the sexual morph of *Arthrobotrys* species, are weak wood decomposers and that nematode cadavers act as an important supply of nitrogen. Predaceous behavior of *A. oligospora* can be controlled either by physiologically active compounds (amino acids or vitamins) present in nematodes or by nitrogen sources.

The majority of nematode-trapping fungi colonize soil, waiting until passing nematodes touch them. Some fungi increase their trapping chances by producing secondary attractive compounds for nematodes, such as *A. superba*, which attracts second-stage juveniles (J2) of *Meloidogyne* species (86). Others grow in the rhizosphere and can even colonize plant roots (76, 80, 87), which gives them superior predatory activity to trap plant-parasitic nematodes on their way to roots. For example, *A. oligospora* was more abundant in the rhizosphere of tomato and barley plants because of its chemotropical attraction to the root tips. Plant species obviously influence the rhizosphere and external root colonization. The highest incidence and diversity of nematode-trapping fungi were detected in association with rhizosphere of peas, while *Dactylellina ellipsospora* and *Arthrobotrys dactyloides* were successfully colonized on tomato roots in a pot experiment (87, 88).

Carbon and nitrogen are two essential nutrients for fungal growth and reproduction. It has been proposed that the nematode-trapping lifestyle is an evolutionary response by cellulolytic or lignin-degrading fungi to nutrient deficiencies in nitrogen-limiting habitats. Because nitrogen is essential for fungal growth and is not freely available in dead wood or in soil where carbon is abundant, direct capture of nitrogen compounds from free-living nematodes or other small animals is advantageous (89). Many network-forming species do not produce a network trap spontaneously, and they are more saprophytic than other nematode-trapping fungi. Formation of network-trapping devices can be induced by the presence of nematodes or substances of animal origin known as nemin. The adhesive network is a primitive characteristic induced only by covering the hypha with a thin film of stick fibrils. Fungal species with other types of trapping devices, such as adhesive knobs (sessile or stalked) and constricting rings, produce trapping devices spontaneously. The spontaneous trap formers more effectively prey on nematodes than do nonspontaneous formers, such as species forming adhesive networks, which have the flexibility to become more predacious by induction of more traps (10).

Various hypotheses on the origin and evolution of nematode-trapping lifestyles in the Ascomycota have been proposed, but conflicts exist between molecular and phenotypic phylogenies (10, 11, 21, 61). The phylogenetic relationships between the different nematode-trapping species need to be known to understand the evolution of parasitism in nematode-trapping fungi. Many molecular phylogenetic studies have shown that the largest group of nematode-trapping fungi belongs to a monophyletic group in the order Orbiliales (Ascomycota). A recent study based on a comprehensive phylogenetic analysis of the nucleotide sequences of three protein-coding genes and rDNA in the internal transcribed spacer region demonstrated that the trapping mechanisms within the Orbiliales have evolved along two major lineages, one leading to species with constricting rings and the other to species with adhesive traps, including three-dimensional networks, knobs, and branches. Among those adhesive trapping devices, the adhesive network separated itself from other trapping mechanisms at an early stage. The adhesive knob evolved through stalk elongation and finally developed into nonconstricting rings. It was shown that the derived adhesive traps are at a highly differentiated stage, but in this study the evolution of the adhesive traps was still ambiguous, partly because the number of taxa representing each type of trapping device was uneven (11).

In a more recent study, data based on a phylogeny of five protein-encoding genes and molecular clock calibration based on two fossil records revealed that fungal carnivorism diverged from saprophytism about 419 million years ago (Mya), which was after the origin of nematodes about 550 to 600 Mya. Active carnivores (fungi with constricting rings) and passive carnivores (fungi with adhesive traps) diverged from each other around 246 Mya, shortly after the occurrence of the Permian-Triassic extinction event about 251.4 Mya. The major adhesive traps evolved around 198 to 208 Mya, which was within the time frame of the Triassic-Jurassic extinction event about 201.4 Mya (Fig. 2). However, more evidence, including additional

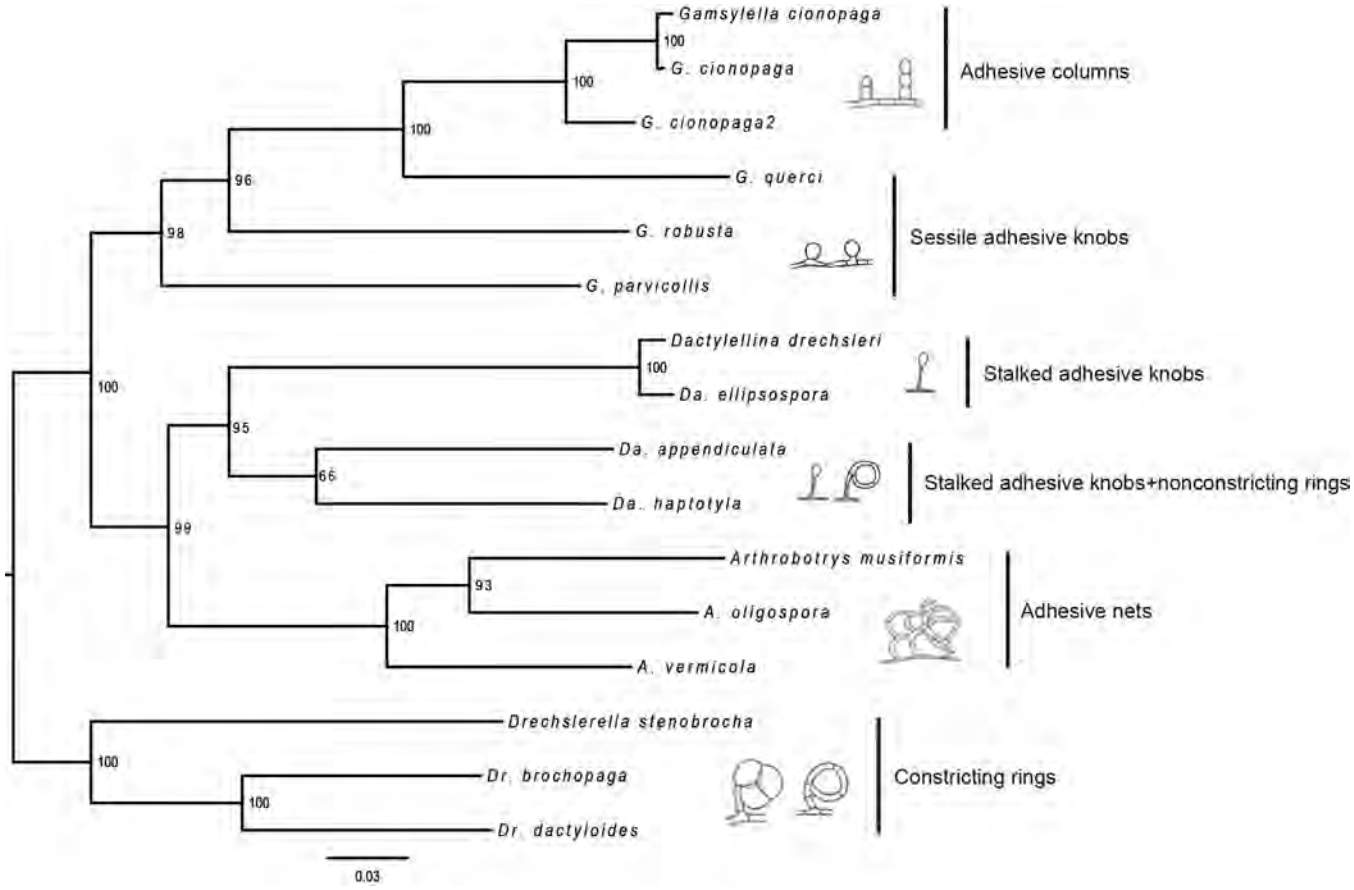


Figure 2 Phylogeny of carnivorous Orbiliomycetes constructed by RAxML using five protein-coding genes. The bootstrap supports are marked on the branch. Trapping devices are drawn on the left.

fossil records, is still needed to illustrate if fungal carnivore evolution was a response to mass extinction events. This study also illustrated a clearer route for the evolution of adhesive traps. Sessile adhesive knobs and an extinct trapping structure (unicellular ring) originated from the adhesive ancestor. Adhesive columns diverged from sessile adhesive knobs by the proliferation of adhesive knobs into a column, which increased the adhesive surface and predatory efficiency. The unicellular ring probably became extinct due to low predatory efficiency (the unicellular ring is morphologically similar to nonconstricting rings, which have the lowest predatory efficiency of existing trapping fungi). The unicellular ring evolved along two lineages to increase predatory efficiency. One formed adhesive nets by proliferating the ring into a network of rings. The other formed nonconstricting rings accompanied by stalked adhesive knobs, in which the single-celled rings developed into three-celled nonconstricting rings, unicellular knobs, and stalked adhesive knobs (10).

MECHANISMS OF FUNGI TRAPPING NEMATODES

Plant-parasitic nematodes cause significant damage to a broad range of vegetables and agricultural crops throughout the world. Understanding the molecular basis of microbe-nematode interactions could lead to the development of more efficient and reliable methods for biological control of parasitic nematodes (12, 90, 91).

Nematode-trapping fungi infect their hosts through a sequence of events (1). They use specialized trapping devices to catch and consume nematodes, and previous studies demonstrated that most fungal species do not produce traps constitutively but, rather, initiate trap formation in response to their prey (92–94). Recognition and adhesion were the first steps in nematode-

trapping fungi infecting their hosts. However, little is known about the molecular mechanisms of recognition and adhesion. Lectin has been reported to be involved in the recognition process (95–97). The finding of lectins in the role of nematode-fungi interaction came from an observation that the interaction between *A. oligospora* and nematodes was mediated by a GalNAc-(*N*-acetyl-D-galactosamine)-specific fungal lectin binding to receptors present on the nematode surface (97). Similar experiments have also indicated that lectins play a role in the adhesion to host surfaces by a number of parasitic and symbiotic fungi (98). However, after a gene encoding such a lectin was deleted in *A. oligospora* by homologous recombination, the deletion mutant showed little decrease in spore (conidia) germination, saprophytic growth, and pathogenicity (99). This result suggested that the fungus might be capable of compensating for the absence of the lectin by expressing other proteins with similar function(s) as the lectin in *A. oligospora*.

The recognition of molecular patterns associated with specific pathogens or food sources is fundamental to ecology and plays a major role in the evolution of predator-prey relationships. Recent studies have shown that nematodes could produce an evolutionarily highly conserved family of small molecules, the ascarosides, which serve essential functions in regulating nematode development and behavior. Interesting research revealed that nematophagous fungi could detect and respond to ascarosides. It was found that ascarosides, which are constitutively secreted by many species of soil-dwelling nematodes, represent a conserved molecular pattern used by nematophagous fungi to detect prey and trigger trap formation. Ascaroside-induced morphogenesis is conserved in several closely related species of nematophagous fungi and occurs only under nutrient-deprived conditions. It was demonstrated that microbial predators eavesdrop on chemical communication among their metazoan prey to regulate morphogenesis, providing a striking example of predator-prey coevolution (37).

Despite large variation in their morphology, adhesive traps share a unique ultrastructure that clearly separates them from vegetative hyphae. One common feature of traps is the presence of numerous cytosolic organelles called dense bodies (100, 101). These organelles always have catalase and D-amino acid oxidase activities, which indicate that they are peroxisome-like organelles. However, the function of these organelles is not yet fully understood. Another feature of traps is the presence of a fibrillar layer of extracellular polymers, which are believed to play an important role in the attachment of the trap cell to the nematode surface (9, 102). Following adhesion, a penetration tube forms and then pierces the nematode cuticle. At this stage, the nematode becomes paralyzed (killed), and the internal tissues are rapidly colonized by fungal hyphae (103).

After recognition and adhesion, the nematode-trapping fungi, like other pathogens, enter into the host through both enzyme degradation and mechanical pressure. The nematode cuticle is composed mainly of proteins including collagen, and several proteases have been isolated from nematophagous fungi that can hydrolyze proteins of the cuticle (104–107). Several extracellular hydrolytic enzymes including serine proteases and collagenases have been detected and partly identified from different nematode-trapping fungi. These studies suggested that extracellular hydrolytic enzymes are key virulence factors involved in the penetration process. Following penetration, the nematode is digested by the invading fungi. These fungi obtain nutrients from the nematodes for their growth and reproduction (108).

APPLICATION OF NEMATODE-TRAPPING FUNGI

In recent decades, concerns about the environmental hazards of using chemical nematicides and limited alternative crops for rotation have led to the development of biological control agents as a component of crop protection. Biological control is considered ecologically friendly and is a possible alternative in pest and disease management. Plant-parasitic nematodes cause significant damage to a broad range of vegetables and agricultural crops throughout the world. Yield losses worldwide to plant-parasitic nematodes have been estimated to range from 5 to 12% annually (3, 4, 6, 109). These losses, however, are influenced greatly by both the production systems and management options utilized. The continuous production of susceptible crops in the sandy soils of the southeastern coastal plains of the United States and other similar regions also often result in devastating losses to nematodes, especially *Meloidogyne* and *Heterodera* species (110).

As the natural enemies of nematodes, nematophagous microorganisms offer a promising approach to control the nematode pests. These biological control agents have an important effect in the regulation of plant-parasitic nematode populations, and numerous organisms including fungi, bacteria, viruses, nematodes, and other invertebrates have antagonistic activity against plant-parasitic nematodes. According to the

report from MarketsandMarkets, from 2009 to the present, the market for biological nematicides has grown nearly 20%, with most of the growth in field crops and biological products occurring in the United States. Sales of nematicides totaled just over $1 billion in 2014 and are expected to grow at a compound annual growth rate of 2.7% in the next 10 years to reach $1.3 billion in 2024. The ability to trap nematodes makes nematode-trapping fungi an attractive candidate agent for controlling parasitic nematodes of plants and animals. Indeed, two commercial biological nematicides, Royal 300 and Royal 350, have been developed based on the species *Arthrobotrys irregularis*, *A. oligospora*, and *Arthrobotrys robusta*. Fungal biological control is an exciting and rapidly developing research area, and growing attention is being paid to the exploitation of fungi to control nematodes (3, 4, 6, 109, 111). The relationship between nematodes and the fungi that infect them has been the subject of widespread mycological studies.

Lots of natural enemies attack nematodes and decrease their populations, but the number of organisms which could be practically employed for biocontrol is restricted (112). In other words, many soil types and their microorganisms all around the world show biological control activity, but their effect on nematode populations can range from insignificant to complete suppression. There have been advances in the biological control of nematodes, but field-scale exploitation of this tactic remains to be realized. Many fungi grow slowly in natural soil, and this can be attributed to inherent competition in the highly diverse communities of soil microfauna and microflora. For a fungus to overcome the competition of resident soil microflora, it must produce a resistant resting stage that is rich in food sources (113). Also, a number of organisms, including mites and possibly mycophagous nematodes, may feed on mycelium of these fungi. Some disadvantages such as complexity in the establishment in the soil, their limited capturing activity, and above all, nonspecific trapping of plant-parasitic nematodes reduce their potential for biological control. Some *Arthrobotrys* species have been formulated and applied under specific conditions, but the results were inconsistent. So there is still a long way to go in developing a highly efficient fungal agent to control nematode pests.

GENOMICS AND PROTEOMICS OF NEMATODE-TRAPPING FUNGI

Genomic and proteomic analyses provided comprehensive understanding of the evolution and infection process of fungi. Recently, the evolution of the nematode-trapping lifestyle and the infection process, including the differentiation of trap cells and the penetration and digestion of nematodes, have been examined using genomic and proteomic approaches.

So far, several genomes of nematode-trapping fungi have been *de novo* sequenced or are currently being sequenced, including *Arthrobotrys conoides*, *A. oligospora*, *Dactylellina appendiculata*, *Dactylellina drechsleri*, *Dactylellina haptotyla, D. stenobrocha*, *Gamsylella cionopaga*, *Gamsylella querci*, and *Orbilia auricolor*. Genomics or proteomics analyses of *A. oligospora* forming adhesive networks (36), *Monacrosporium haptotylum* forming adhesive knobs (35), and *D. stenobrocha* forming constricting rings (33) have been published and provided a new approach for obtaining a better understanding of the interaction between nematode-trapping fungi and their hosts.

Molecular phylogenies based on rDNA sequences strongly support that the nematode-trapping lifestyle evolved once in the Orbiliales. Subsequently, several distinct trapping types evolved. On the genomic level there are three compatible mechanisms that could account for such evolutionary patterns. First, parasitism has been associated with the presence of novel genes. Such genes could be acquired by gene duplication or horizontal gene transfer. Second, adaptations to the parasitic habit may result from the differences in the regulation of gene expression. Third, parasitism is associated with gene loss and deletions (114). By comparative genomics analysis of the knob-forming species *M. haptotylum* and the net-forming species *A. oligospora*, two genomic mechanisms have been determined to be important for the parasitic adaptation in these fungi. First, the expansion of protein domain families and the large number of species-specific genes indicated that gene duplication followed by functional diversification had a major role in the evolution of the nematode-trapping fungi. Gene expression indicated that many of these genes are related to pathogenicity. Second, the gene expression of orthologs between the two fungi during the infection process indicated that differential regulation was an important mechanism for the parasitic evolution in nematode-trapping fungi (35).

Comparative genomics analysis showed that *A. oligospora* shared many more genes with pathogenic fungi than with nonpathogenic fungi. Specifically, compared to several sequenced ascomycetous fungi, the *A. oligospora* genome has a larger number of pathogenicity-related genes in the subtilisin, cellulase, cellobiohydrolase, and pectinesterase gene families. Searching the pathogen-host interaction gene database identified 398

homologous genes involved in pathogenicity in other fungi. The analysis of repetitive sequences provided evidence for repeat-induced point mutations in *A. oligospora*. Proteomic and quantitative PCR analyses revealed that 90 genes were significantly upregulated at the early stage of trap formation by nematode extracts, and most of these genes were involved in translation, amino acid metabolism, carbohydrate metabolism, and cell wall and membrane biogenesis. Based on the combined genomic, proteomic, and qPCR data, a model was proposed for understanding the formation of the nematode-trapping device in this fungus. In the model, multiple fungal signal transduction pathways are activated by its nematode prey to further regulate downstream genes associated with diverse cellular processes such as energy metabolism, biosynthesis of the cell wall and adhesive proteins, cell division, glycerol accumulation, and peroxisome biogenesis (35, 36).

The trap of *M. haptotylum* consists of a unicellular structure called a knob that develops at the apex of a hypha. To better understand the cellular functions of adhesive traps, the trap cell proteome of the fungus *M. haptotylum* was characterized. In this research, 336 proteins were identified, with 54 expressed at significantly higher levels in the knobs than in the mycelia. The upregulated knob proteins included peptidases, small secreted proteins with unknown functions, and putative cell surface adhesins containing carbohydrate-binding domains, including the WSC (cell wall integrity and stress-response component) domain. A further phylogenetic analysis showed that all upregulated WSC domain proteins belonged to a large, expanded cluster of paralogs in *M. haptotylum*. Several peptidases and homologs of experimentally verified proteins in other pathogenic fungi were also upregulated in the knob proteome. Complementary profiling of gene expression at the transcriptome level showed poor correlation between the upregulation of knob proteins and their corresponding transcripts. It was concluded that the traps of *M. haptotylum* contain many of the proteins needed in the early stages of infection and that the trap cells can tightly control the translation and degradation of these proteins to minimize the cost of protein synthesis (115).

These genomic and proteomic studies will greatly facilitate the identification of pathogenicity-related genes and provide a broad foundation for understanding the molecular and evolutionary mechanisms underlying fungal-nematode interactions.

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Host-Microsporidia Interactions in *Caenorhabditis elegans*, a Model Nematode Host

48

Emily R. Troemel

NEMATOCIDA SPECIES ARE NATURAL MICROSPORIDIAN PATHOGENS OF THE NEMATODE *C. ELEGANS*

The nematode *Caenorhabditis elegans* is a highly tractable organism that has been studied in the laboratory for several decades and has provided novel insights into questions of development, neurobiology, and cell biology, among other basic biological processes (1). Until recently, however, little was known about its natural ecology. Now, because of greatly increased sampling of *C. elegans* from the wild, we are learning more about its life outside the laboratory (2). In particular, it has become clear that *C. elegans* and other *Caenorhabditis* nematodes are commonly infected by microsporidia in the wild (3–5). Microsporidia comprise a phylum of over 1,400 species of fungal-related parasites that can infect nearly all animal hosts (6–8), so it is perhaps no surprise that these pathogens commonly infect the nematode *C. elegans*.

Microsporidia have been studied since the time of Louis Pasteur, and they have a very specialized obligate intracellular lifestyle that shares some similarities with protozoan parasites (Fig. 1) (6). The stage of microsporidia that survives outside the host cell is the dormant spore stage, which is characterized by a specialized infection apparatus called a polar tube. This tube is coiled inside the spore until it receives the proper cue, at which point it fires the tube and injects a parasite cell directly into the host cell. This cell then replicates intracellularly as a meront, which in most species is a multinucleated cell. Meronts eventually differentiate back into spores that are released from the host to transmit infection. In addition to this unique intracellular life cycle, microsporidia are distinguished by a lack of true mitochondria and smaller ribosomal rRNA genes, which led them to initially be proposed as ancient eukaryotes. However, with more genome sequence available, it has become clear that microsporidia are either close relatives of fungi, or true fungi (9, 10). Notably, microsporidia have the smallest known eukaryotic genomes, with the human-infecting species *Encephalitozoon intestinalis* genome only 2.3 Mb (11), which is smaller than many bacterial genomes.

ANALYSIS OF THE GENOMES FROM *NEMATOCIDA* AND OTHER MICROSPORIDIA SPECIES

The first and best-described microsporidian pathogen of *C. elegans* was obtained from a wild-caught *C. elegans* isolated from a compost pit near Paris, and it was named *Nematocida parisii* (nematode killer from Paris) strain ERTm1 (3). *N. parisii* causes a lethal intestinal infection in *C. elegans* (3), and it can also cause developmental arrest of *C. elegans* larvae (12). In the original study, two other *N. parisii* strains from wild-caught *C. elegans* in France were described (strains ERTm3 and ERTm4), as well as a related *Nematocida* species called *Nematocida* sp. 1 (strain ERTm2), which was found in a sister species of *C. elegans* called *Caenorhabditis briggsae* isolated from a park in India (3). Genome sequencing of *N. parisii* strain ERTm1 demonstrated a genome size of 4.1 Mb, containing 2,661 predicted genes, with expression of most of these genes confirmed by transcriptome analysis of several stages of the *N. parisii* life cycle (10). The *N. parisii* isolate ERTm3 genome had a similar size and predicted gene set, while an isolate of the sister species *Nematocida*

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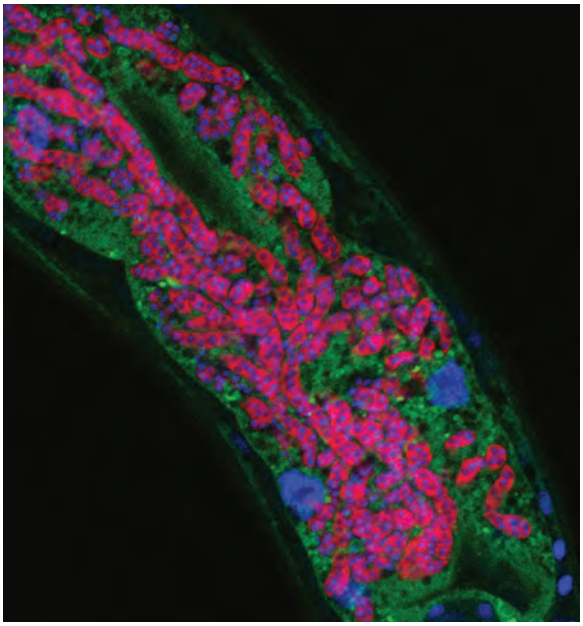


Figure 1 *N. parisii* microsporidia infecting the *C. elegans* intestine at the meront stage. *N. parisii* meronts are labeled with a fluorescent probe for rRNA in red, the *C. elegans* intestine is labeled with cytoplasmic GFP in green, and DNA is labeled with DAPI in blue. DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein. (Image credit Susannah Szumowski.)

sp. 1 sequencing revealed a slightly larger genome of 4.7 Mb and 2,770 predicted genes. Thus, like other microsporidia species, *Nematocida* genomes are quite small, being smaller than many bacterial genomes.

Interestingly, genome sequencing indicated that *Nematocida* are diploid—the ploidy was unknown for any microsporidia species before this study. Most regions of the *Nematocida* sp. 1 genome were highly heterozygous, with one single nucleotide polymorphism every 82 bp (10). *N. parisii* was similarly found likely to be diploid, although with much less heterozygosity than *Nematocida* sp. 1 (strain ERTm2). Genome sequencing of a second *Nematocida* sp. 1 strain (ERTm6) isolated from a *C. briggsae* in Cape Verde indicated that it also is heterozygous, although not as heterozygous as ERTm2 (13). Subsequent studies of other microsporidia species have indicated that they too are diploid (14). Intriguingly, several chromosomal arms in *Nematocida* strains have a loss of heterozygosity (LOH), indicating there has been a rare or recent recombination event, consistent with a sexual cycle for microsporidia (10). Rare sexual cycles have been shown to be important for virulence in other fungal species (15). Passaging of *Nematocida* sp. 1 for 3 months in the laboratory did not result in a change in the pattern of LOH, suggesting that a sexual cycle did not occur under these laboratory conditions (10). It would be interesting to examine the patterns of LOH under more natural conditions to determine if or when a sexual cycle may occur for *Nematocida* species, and whether it is linked to virulence.

Using the *Nematocida* genome sequences described above, together with genome sequences of other microsporidian species that infect insects and humans, comparative analysis was performed to gain a global view of the microsporidia phylum (10). In particular, ten microsporidian genomes were analyzed to determine which genes were gained and lost in comparison with "true fungi." This phylogenomic analysis uncovered potential cancer-like strategies that microsporidia may use to replicate rapidly in host cells. These studies showed that all microsporidia have lost the retinoblastoma gene (RB), which encodes a cell cycle inhibitor lost in most human cancers. Evolutionary loss of RB perhaps enabled microsporidia to acquire a fast cell cycle to replicate quickly and outpace the host in the utilization of resources. Loss of RB should also lead to a mistake-prone cell cycle and accumulation of mutations, which might explain why microsporidia have "long branches" in phylogenetic trees. This long-branch length has led to controversy in the phylogenetic placement of microsporidia, but data from this study and another study analyzing 53 shared genes have firmly established microsporidia as the earliest branching group of the sequenced fungi (9, 10). Intriguingly, it was found that microsporidian hexokinases have gained secretion signal sequences, and in a functional assay these were sufficient to export proteins out of the cell; thus, hexokinase may be targeted into the host cell to reprogram it toward biosynthesis, similar to the "Warburg effect" in cancer cells (10). This study, together with previous studies (16, 17), showed that microsporidia horizontally acquired transporters that could import nucleotides and nucleosides to fuel their rapid growth. Thus, rapid growth using cancer-like strategies, together with a rare sexual cycle for diversity, provide potential explanations for the emergence and success of these diverse intracellular parasites (10).

Bolstered by the *Nematocida* genome data described above, *C. elegans* provides a convenient whole-animal host to study microsporidia infection because of its transparent body plan and the ease of culturing it in the laboratory. In particular, it provides an attractive model for intestinal infection, because its cells share similar structural features with human intestinal epithelial cells, with apically polarized microvilli anchored into a cytoskeletal structure called the terminal web (18–

20). In the laboratory, *Nematocida* can proliferate to occupy the entire intestine of *C. elegans*, which can be observed in intact animals by staining with fluorescence *in situ* hybridization probes for parasite rRNA (Fig. 1). Because of the convenient culturing and imaging of *C. elegans*, together with the powerful genetic and molecular tools, it has been possible to molecularly characterize different aspects of its life cycle in the intestine of live animals to determine how it molecularly interacts with the host; these studies are described below.

N. PARISII RESTRUCTURES *C. ELEGANS* INTESTINAL CELLS AND EXITS VIA APICAL EXOCYTOSIS

N. parisii infection causes extensive cytoskeletal restructuring in the *C. elegans* intestine as part of its exit process (21). Like most microsporidia, *N. parisii* survives outside of host cells in the transmissible spore form, and replicates exclusively inside of host cells in a form called a meront (3). Early during infection, when *N. parisii* is replicating inside the cell as a meront, there is a partial loss of cytoskeletal polarity of *C. elegans* intestinal cells (Fig. 2). Normally, the intestinal-specific isoform of actin called ACT-5 is restricted to the apical side of the cell. However, *N. parisii* infection at the meront stage causes ectopic expression of this host ACT-5 to the basolateral side of the cell (22). Soon after this redistribution of actin, gaps appear in the terminal web, which is a conserved cytoskeletal structure composed of actin and intermediate filaments (3, 22). This redistribution of actin may in fact trigger the formation of terminal web gaps, because reducing actin expression with RNA interference caused the formation of terminal web gaps in the absence of infection (22). Characterization of terminal web gaps indicated that they appear precisely when spores begin to form and exit from the cell. Once terminal web gaps form, there do not appear to be additional gaps that form later, although the existing gaps become larger probably because of distension of the lumen. These gaps appear to be specific to *N. parisii* infection, in that they do not appear in response to infection with other pathogens that cause lethal intestinal infections in *C. elegans*. Although the exact function of these gaps has not been established, it has been proposed that they serve to remove a physical barrier to exit for *N. parisii*, such that the spores are able to traverse this cytoskeletal structure to reach the apical surface of the intestinal cell for exit into the lumen.

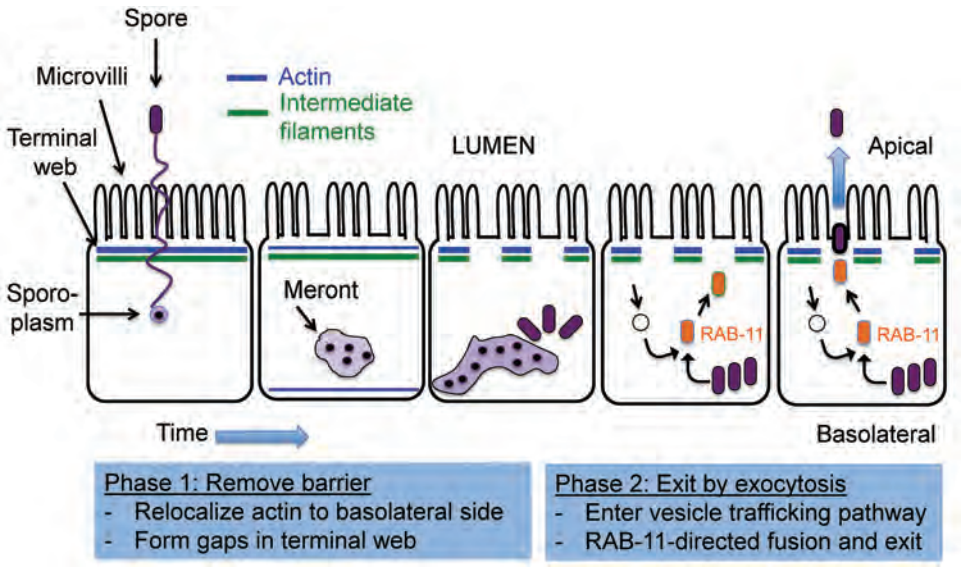


Figure 2 *N. parisii* restructures *C. elegans* intestinal cells and exits via RAB-11-directed apical exocytosis. *N. parisii* exit from *C. elegans* intestinal cells is a two-phase process. Phase 1: When *N. parisii* replicates as a meront inside *C. elegans* intestinal cells, the actin isoform ACT-5 is no longer restricted to just the apical side and instead appears to be ectopically expressed on the basolateral side of the cell. This relocalization may trigger gaps in the terminal web that occur just as spores begin to form in the intestinal cell. Phase 2: *N. parisii* spores are found in separate membrane-bound compartments, become coated in the host small GTPase RAB-11, which is required for spore-containing compartments to fuse with the apical membrane and allow spores to exit into the lumen.

Restructuring of the terminal web may remove a barrier to exit for *N. parisii* so it can reach the apical surface, but how does this pathogen actually traverse this membrane to exit from host cells? Initial studies of *N. parisii* indicated there might be a regulated form of exit, because animals infected with just a few spores were contagious to their neighbors (3). This transmission could happen from animals that were alive and seemingly healthy, suggesting that intestinal cells were not lysed to release pathogen. This nonlytic exit was confirmed through the use of cell integrity assays, and analysis of green fluorescent protein-labeled intestinal cells suggested that the exit was not due to budding off of membranes (22). Because *C. elegans* only has 20 nonrenewable intestinal cells, it is likely advantageous for *N. parisii* to exit from these cells nonlytically.

Further analysis with electron microscopy and molecular markers indicated that, after *N. parisii* meronts differentiate into spores, they are found in a separate membrane-bound compartment, suggesting a membrane fusion mode of exit (23). Indeed, using a marker for the apical side of intestinal cells it was demonstrated that *N. parisii* spore-containing compartments can fuse with the apical membrane and be released into the intestinal lumen to be defecated out and spread to new hosts for disease transmission (23). This exit appears to be highly directional, with exit only out of the apical side into the lumen with no evidence of exit basolaterally to spread into other tissues of the animal (22). Furthermore, this exit is prodigious, with 1 to 2,000 spores shed per worm per hour for several hours (22). Using a spore-shedding assay, researchers screened a small library of predicted small GTPases in *C. elegans* and identified the small GTPase RAB-11 as a critical host factor required for spore fusion, spore exit, and host contagiousness (23). RAB-11 localizes extensively to spores poised to exit into the intestinal lumen. Because RAB-11 directs recycling endocytosis to the apical side of the cell, by hijacking this pathway *N. parisii* can accomplish directional exit from the cell without causing a lysis event that would likely be very detrimental to its host.

In addition, there has been a recent report of the formation of actin coats around exocytosing spores (24). In this study, a genetic screen was performed to identify small GTPases required for formation of these coats, and hits included known actin regulators including Rac1/CED-10 and Cdc42/CDC-42. Analysis of CED-10- and CDC-42-defective animals, which lack these actin coats, indicated that actin coats are not required for spores to exit. However, it is possible that they facilitate exit, perhaps acting to stabilize the large spore cargo contained in an exocytic vesicle. Later during infection there are also large vesicles coated with actin that contain many *N. parisii* spores, and analysis indicates that these vesicles are due to compensatory endocytosis to maintain the membrane balance after the extensive exocytosis of *N. parisii* spores (24).

C. ELEGANS EXHIBITS A UBIQUITIN-MEDIATED RESPONSE TO *N. PARISII* INFECTION

In order to describe the *C. elegans* host response to *N. parisii* infection, RNAseq analysis was performed at five time points during *N. parisii* infection (25). These studies demonstrated that the *C. elegans* transcriptional response to this natural intracellular pathogen is distinct from responses to other previously described extracellular pathogens. However, there was a very strong similarity in the gene expression response to a natural viral pathogen, indicating that some similar feature of the infection caused by these two very distinct pathogens is eliciting a transcriptional response in the host (25, 26). This response was characterized by an upregulation of ubiquitylation components, and these had a functional role in protecting the host from infection, together with components of the autophagy pathway (25). For example, if components of the autophagy pathway were reduced in expression with RNAi, then animals were more susceptible to infection, whereas if the autophagy pathway was upregulated, then animals were more resistant to infection. Furthermore, there was localization of ubiquitin and autophagy components to meronts, suggesting that they are being targeted for destruction by ubiquitin-mediated autophagy, which is a common host defense strategy against intracellular pathogens. Interestingly, many of the microsporidia and virus response genes are also induced by inhibition of the ubiquitin-proteasome system (25). Thus, a trigger for transcriptional response to intracellular infection caused by microsporidia and virus may be perturbation of the ubiquitin proteasome system.

NATURAL VARIATION IN HOST RESISTANCE TO MICROSPORIDIA

Although the studies described above indicate that ubiquitin-mediated signaling in the N2 laboratory strain of *C. elegans* can provide some resistance against *N. parisii*, this host strain always ultimately succumbs to *N. parisii* infection. Interestingly, there is natural variation in *C. elegans* resistance against *N. parisii*, with a *C. elegans* strain from Hawaii that has the ability

to clear infection, demonstrating striking immune capabilities for epithelial cells (27). This capability is restricted to very young animals, and is lost rapidly as animals develop, even before attaining reproductive stage. Intriguingly, it is only at this young age that microsporidia impact the reproductive output of animals, and thus, from an evolutionary perspective, this is the most important life stage at which to have pathogen resistance. With quantitative genetics it was shown that this enhanced, early-life resistance of Hawaiian animals is a complex trait comprising at least four distinct genetic loci. By using near-isogenic lines it was confirmed that two of these loci confer resistance and act additively (27). Although the genes responsible for this resistance have not yet been identified, there are several exciting candidates in these regions, including F-box genes that can serve as ubiquitin ligase adaptors. Of note, these F-box genes are among the most rapidly evolving genes in the *C. elegans* genome, which might be a sign that they are part of an interface with a rapidly evolving pathogen, as part of a host/pathogen coevolutionary arms race (28). Further studies will investigate this possibility. This enhanced resistance of the Hawaiian strain of *C. elegans* enables them to outcompete the susceptible laboratory strain in just a few generations with selective pressure from pathogens. Altogether these findings indicate impressive abilities of epithelial cells in this wild *C. elegans* strain to clear intracellular infection by microsporidian pathogens.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The use of *C. elegans* as a model host for microsporidian infection has provided insight into the exit strategies of microsporidia and how they exploit host cell cytoskeletal and trafficking pathways to facilitate their life cycle. These studies have also provided insight into defense pathways and natural variation in host resistance. Sequencing of the genomes of several microsporidia species has provided intriguing hints about the evolutionary steps that have led to the emergence of this phylum, and identified several candidate effectors these pathogens use to manipulate hosts. However, studies of microsporidia infection in *C. elegans* and any other host are hampered by the lack of ability to transform and genetically manipulate these parasites. Developing these tools would greatly facilitate our understanding of the pathogenesis and life cycle of these ubiquitous parasites.

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Bacterial Endosymbionts: Master Modulators of Fungal Phenotypes

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INTRODUCTION

From the saprotrophs that decay plant material to the pathogens and mutualists that shape plant population dynamics at local and regional scales, fungi are major drivers of ecosystem health, plant productivity, and sustainability in all major biomes (1–5). The ecological roles of such fungi are driven by their own genomic and epigenetic architecture, as well as that of their hosts, often with strong influences from the environmental context in which such interactions occur (1–9).

The environmental context of fungal biology has long been defined in terms of abiotic factors, including those that impact fungal growth and gene expression directly and those that influence the health, growth, structure, or defenses of hosts or substrates with which fungi interact (Fig. 1). Increasingly, however, it is clear that these environmental contexts also include biotic components—that is, additional, interacting species that directly or indirectly alter the outcomes of fungus-host associations. Foremost among these in terms of major effects are endohyphal microbial symbionts, including viruses and bacteria that occur within living fungal hyphae and can profoundly alter fungal phenotypes (i.e., endofungal microbes or microbial endosymbionts) (10–13).

Phenotypic modulation of fungi by endohyphal microbes can cause major shifts in fungal substrate use, enzyme production, thermotolerance, virulence, and the early phases of symbiotic establishment. Much like horizontal gene transfer (HGT) can alter diverse aspects of an organism's phenotype in ecological time, so too can acquisition (or loss) of a microbial symbiont change a virulent fungal pathogen to an avirulent lifestyle, alter the reproductive strategy of the fungus from sexual to asexual, increase fungal secretion of hormones and other signaling molecules relevant to plants, or broaden the niche space and competitive ability of fungi in ways that change the balance of fungal community dynamics (see references 11–19). In these ways endohyphal microbes are important not only in ecological time, but also in the evolution of fungi and reciprocal coevolutionary responses from their hosts (Fig. 1). Thus, understanding the mechanisms underlying symbiotic modulation of fungal phenotypes by microbes has the potential to inform not only fungal ecology but also the trajectory of fungal evolution, especially with regard to convergent phenotypes or ecological modes (e.g., endophytism) for which genomic markers and evolutionary shifts remain elusive. Such questions, paired with growing interest in applications of microbial endosymbionts of fungi, frame an exciting time as mycologists define myriad factors that shape fungal functional traits in short-term ecological snapshots and over evolutionary time.

Broadly defined under de Bary's conceptualization as "the living together of unlike organisms" (20), symbioses between fungi and endohyphal microbes encompass mutualistic, parasitic, and commensal interactions, many of which may be context-dependent in terms of their importance or effects. The best known microbes that occur as endohyphal symbionts represent two domains of life. The first are acellular microbes such as mycoviruses. Mycoviruses (viruses that infect fungi) usually are double-stranded RNA viruses (ca. 70% of known mycoviruses), positive-sense, single-stranded RNA viruses (ca. 30%), and rarely, geminiviruses (21).

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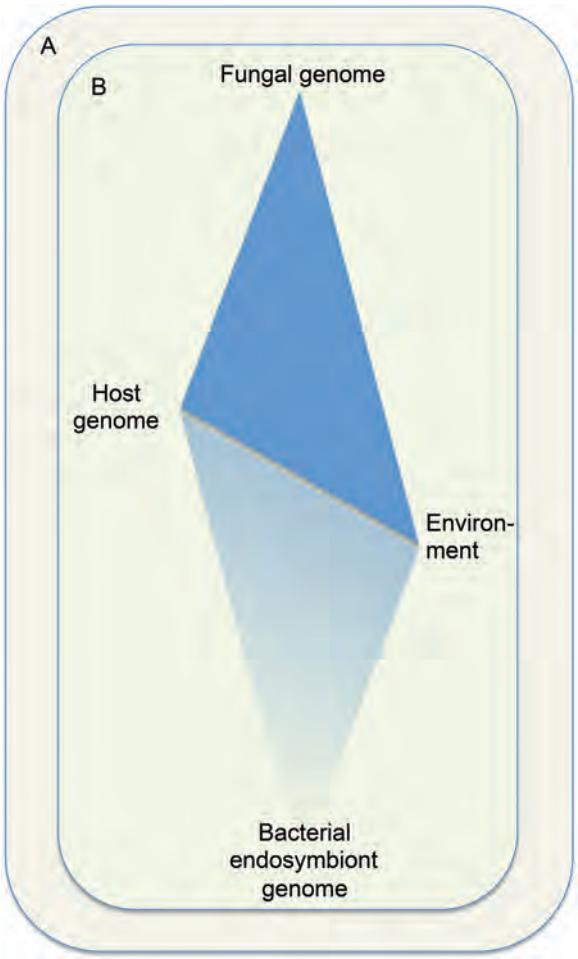


Figure 1 Fungal phenotypes result from direct and indirect interactions of fungal genomes, host genomes, substrate characteristics, and the environment, as well as the genomes of EHB. These often dynamic interactions occur in the context of biotic communities (**A**) that are shaped by an evolutionary and biogeographic context (**B**).

Viruses clearly are important in fungal biology (22–25). Their effects include hypovirulence of pathogens such as *Cryphonectria parasitica*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Fusarium graminearum* (26–30); disease in various commercially important Agaricomycetes (31, 32); thermotolerance of plants via infection of the fungal endophyte *Curvularia protuberata* (33); and interference with the reproduction of other mycoviruses (34). Studies over the past 50 years have revealed that mycoviruses are ubiquitous in fungi (35, 36), with modes of transmission primarily via hyphal anastomoses and both sexual and asexual spores (but see reference 37 for an example of transmission via a mycophagous insect). Mycoviruses and their importance in fungi represent an exciting frontier for future research relevant to all aspects of fungal biology, and the themes structuring such research (i.e., effects on host phenotypes and gene expression, interaction with cellular defenses, modes of transmission, and roles in fungal ecology and evolution) are being investigated in parallel with studies of the other well-documented and diverse endosymbionts of fungi, in the domain Bacteria.

Much like other eukaryotes, fungi interact closely with bacteria. These interactions occur in the mycelosphere (the area around the mycelium with which the fungus interacts), on living hyphae (i.e., on the mycelo-plane), and within living structures such as hyphae (and in some cases spores; reviewed in reference 16). Those bacteria that occur within living hyphae are endofungal bacteria or endohyphal bacteria (EHB), broadly parallel to the concept of fungi occurring as endophytes of plants. These bacteria are grouped functionally based on their observation within hyphae, but their names do not imply obligate endosymbiotic lifestyles or proscribed benefits or costs to hosts. Instead, these phylogenetically diverse bacteria appear to have converged upon the capacity to infect fungal hyphae (38), together encompassing a spectrum of facultative to obligate associations, diverse localizations within fungal cells, and a range of outcomes of their associations.

Our thesis is that understanding the contributions of these EHB to fungal biology is a major frontier in studies of the fungal kingdom (16, 38–42). Their study is particularly exciting at present given the potential to explore new fungal strains and well-established model systems with high-throughput phenotyping and front-line molecular and computational methods in metabarcoding, genomics, and metatranscriptomics (19, 41–44). Our goals in this chapter are to provide a historical perspective on EHB as a means to frame a functional classification system for these bacterial associates of fungi, to summarize their functional importance and related genomic traits, and to highlight emerging methods by which EHB can be studied, pointing to emergent questions regarding the role of bacterial endosymbionts in fungal biology.

HISTORICAL PERSPECTIVES

Often present in symptomless mycelia, EHB inhabit diverse fungi representing at least the Mucoromycota (*sensu* reference 45: Mortierellomycotina, Mucoromycotina, Glomeromycotina) and Dikarya (Basidiomycota and Ascomycota) (38, 46–49). Most fungi documented as hosts of EHB are plant-associated, including arbuscular and ectomycorrhizal fungi, plant pathogens, saprotrophic fungi, soilborne fungi that interact with seeds,

and endophytes of tissues such as roots and leaves. Some clinically important fungi also harbor EHB, though they do not appear to play a major role in pathogenesis in human and animal systems (50–52).

Although it is not surprising that like many eukaryotes, fungi host bacterial endosymbionts, the discovery of their prevalence and their potential to shape major plant-fungi interactions has captured recent interest. The seminal discovery in 2005 that pathogenicity of a major plant pathogenic fungus, *Rhizopus microsporus*, was driven by an EHB (14) brought classical research on "bacteria-like organelles" (BLOs) (53) to the fore of plant pathology and mycology alike. Now, bacterial genomics, microscopy, and molecular biology are providing insights into the endohyphal lifestyle and the mechanisms by which such bacteria shape fungal traits.

EHB were first described in arbuscular mycorrhizal fungi in the 1970s (54, 55), although their status as bacteria was not confirmed until they were imaged by electron microscopy (53). Their discovery was complemented by the description of the cyanobacterium *Nostoc punctiforme* living within the fungus *Geosiphon pyriforme* (Glomeromycotina) (see 15, 56–59). Most classical mycology texts rarely mention bacteria except to differentiate them from fungi (e.g., 60–63, as also observed in reference 64). However, Wolf and Wolf frequently reference bacteria-fungi interactions, referencing "associative interactions" and characterizing fungi as gregarious in terms of their associations with prokaryotes (65). Notably, Wolf and Wolf also note that "pure cultures" of fungi in nature are "either non-existent or else occur as miraculous oddities" (65), speaking presciently to our current understanding of the complex associations that frame many aspects of microbial and fungal ecology.

In the 1970s several studies established that BLOs were present in diverse fungi. Mosse noted the presence of self-replicating bacteria in spores of *Endogone*, speculating that BLOs observed there might be spores of Actinobacteria (66). Protzenko also observed a bacterium or BLO in an endotrophic mycorrhizal fungus of pea, noting that the microbe's cell wall was located in close proximity to that of its host and stating that no morphological response of the fungus was observed (54). Microbial associates were observed in Ascomycota as early as 1973, when intracytoplasmic organisms ("probably bacteria") were recorded in *Scutellinia*, a genus of saprotrophs (67).

In most of these cases early observations were by microscopists, with MacDonald et al. noting that a major challenge in the further study of BLOs was that they appeared to be unculturable (68). Perhaps for this reason studies contemporaneous with the early description of BLOs focused on other bacteria-fungi associations despite compelling evidence of unusual microbial- or microbe-like structures in fungal hyphae. For example, the last decades of the 20th century were particularly rich in studies regarding mycorrhizal helper bacteria (reviewed in reference 69; see also reference 16), bacterial ectosymbionts of fungi (70, 71), interactions between cyanobacteria and lichen-forming fungi (72), bacterial pathogens of cultivated mushrooms (73, 74), the importance of mixed-microbial fermentations in foods (64, referencing 75), and antagonism between fungi and bacteria. Scannerini and Bonfante-Fasolo summarized the state of the field with respect to BLOs and endosymbiotic bacteria in fungi in general: "the available information remains scanty" (76).

Even so, Scannerini and Bonfante-Fasolo pointed to ca. 10 published and unpublished studies highlighting the structural classification of BLOs and endosymbiotic bacteria in arbsucular mycorrhizal fungi, providing evidence that these "endocytobionts" could be observed in spores, hyphae, arbuscules, and other structures (76). Moreover, they diagnosed the existence of two types of endocytobionts in fungal tissue: (i) rod-shaped bacteria with visible cell walls, nucleoids, and ribosomes, typically enclosed in vacuoles, observed in *Glomus* and an unidentified arbuscular mycorrhizal fungus (68, 77, 78), and (ii) BLOs, which lack cell walls, have a coccoid form, and are localized in the cytoplasm of diverse arbuscular mycorrhizal fungi. MacDonald and Chandler reported that such BLOs typically could be found near the nucleus or mitochondria of fungal cells, although their placement varied (53). Scannerini and Bonfante-Fasolo concluded that these BLOs resemble the vertically transmitted bacterial endosymbionts of some insects (76).

Enabled by these pioneering studies, a groundswell of interest in symbioses, and the combination of new analytical methods in visualization and molecular biology, a renewed focus on bacterial endosymbionts of fungi has emerged over the nearly 3 decades since Scannerini and Bonfante-Fasolo's review (76). Reconstruction of this phase in the study of bacterial endosymbionts is somewhat challenging because terminology has changed over time (i.e., endocytobionts, endocellular bacteria, endofungal bacteria, endobacteria, bacterial endosymbionts and endosymbiotic bacteria, and EHB). However, literature searches (e.g., in the Web of Science, early 2017, with these terms in Boolean arrays) point to a growth in publication rate from 1996 onward, especially over the past 10 years.

In 1996, one paper highlighted the presence of ca. 250,000 BLOs in a single spore of the arbuscular mycorrhizal fungus *Gigaspora margarita* and used 16S rRNA sequencing to identify bacteria within living fungi as members of the Gram-negative bacterial genus *Burkholderia* (*Betaproteobacteria*; now recognized as *Paraburkholderia* and hereafter referred to as such [46, 79]). By 2000, such studies included surveys of diverse arbuscular mycorrhizal fungi and revealed phylogenetic relationships among *Paraburkholderia* associated with various Gigasporaceae (80). Tools such as *in situ* hybridization further enabled visualization of symbiotic bacteria in spores and hyphae (e.g., of *G. margarita* [80]). Subsequent studies moved rapidly to address the functional significance of such bacteria, drawing from genomics to show that a *Paraburkholderia* in *G. margarita* has nitrogen fixation genes (81) and identifying regions coding for particular proteins (e.g., a methyl accepting chemotaxis protein [82]).

The first major papers on other fungal systems emerged with the 2005 study of *R. microsporus* (Mucoromycotina), wherein a strain of *Paraburkholderia* was shown to produce rhizoxin, a macrocyclic polyketide metabolite and phytotoxin critical to pathogenicity associated with rice seedling blight (14). By 2010, endohyphal *Burkholderiaceae* also were reported from Mortierellomycotina (83), highlighting the prevalence of this bacterial family across the Mucoromycota. Contemporaneously, *Alphaproteobacteria* were identified in plant-associated mycelium of the ectomycorrhizal basidiomycete *Laccaria bicolor* (84), demonstrating that diverse lineages of *Proteobacteria* can occur as endofungal symbionts and indicating that septate hyphae such as those in the Basidiomycota did not preclude establishment of EHB. A second *Alphaproteobacteria*, *Rhizobium radiobacter*, was identified subsequently as relevant to the establishment of endophytic symbioses by the basidiomycete *Piriformospora indica* (Sebacinales), which affiliates with roots of barley and other hosts (85).

Since that time, studies of EHB have widened in two main ways. First, the combined approaches of molecular biology, genomics, and computational biology have led to the identification of the coccoid BLOs in Glomeromycotina that had been observed for decades by microscopy alone. Now known as *Mollicutes*-related endobacteria and placed phylogenetically in the *Tenericutes*, these EHB are recognized as widespread symbionts of arbuscular mycorrhizal fungi (86, 87). They exhibit within-host genetic variation, small genomes, and complex genomic and evolutionary histories (41, 42, 88).

Second, the study of EHB now has extended to the Ascomycota, the most species-rich phylum of fungi. In the first studies evaluating phylogenetically diverse Ascomycota for EHB, fungi occurring as foliar endophytes were shown to harbor not only many lineages of *Proteobacteria* but also diverse Gram-negative and Gram-positive bacteria from the *Bacteroidetes* and *Firmicutes*, respectively (38, 49). As in other groups of fungi, visualization of these EHB was facilitated by LIVE/DEAD staining (i.e., LIVE/DEAD BacLight Bacterial Viability Kit, Invitrogen [38]) and by more precise methods such as fluorescent *in situ* hybridization (Fig. 2) and bacterial transformation for fluorescence (Fig. 3). Recent work has addressed the phylogenetic diversity of EHB (Fig. 4) (see 49), their significance with regard to fungal thermotolerance, production of plant hormones and activity of plant cell wall-degrading enzymes (Fig. 5) (11, 17), their alteration of fungal growth and plant tissue degradation (Fig. 6), their capacity to influence early stages of symbiotic establishment between endophytes and hosts (Fig. 7), their variation among closely related fungi that infect seeds as opposed to leaves (49), their ability to alter carbon substrate use by a

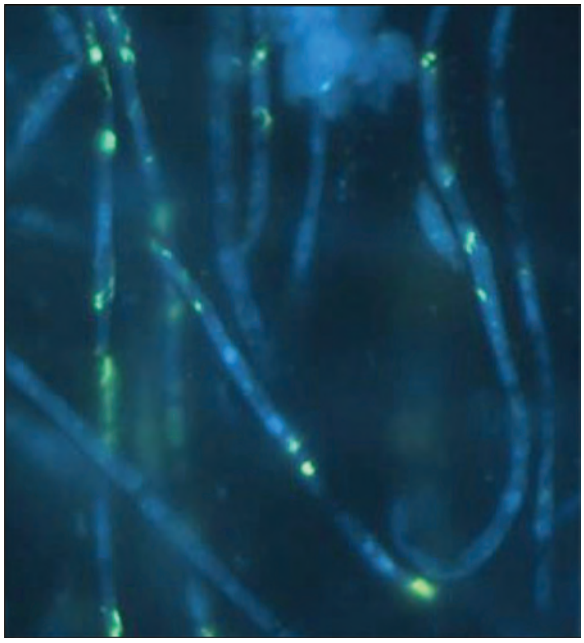


Figure 2 Fluorescent *in situ* hybridization microscopy showing endophytic *Pestalotiopsis* sp. 9143 harboring the class 3 endohyphal bacterium *Luteibacter* sp. 9143. The image shows the TAMRA fluorophore with the DAPI counterstain (blue), highlighting fungal nuclear and mitochondrial DNA in addition to bacteria (yellow/green). (Reprinted from reference 38).

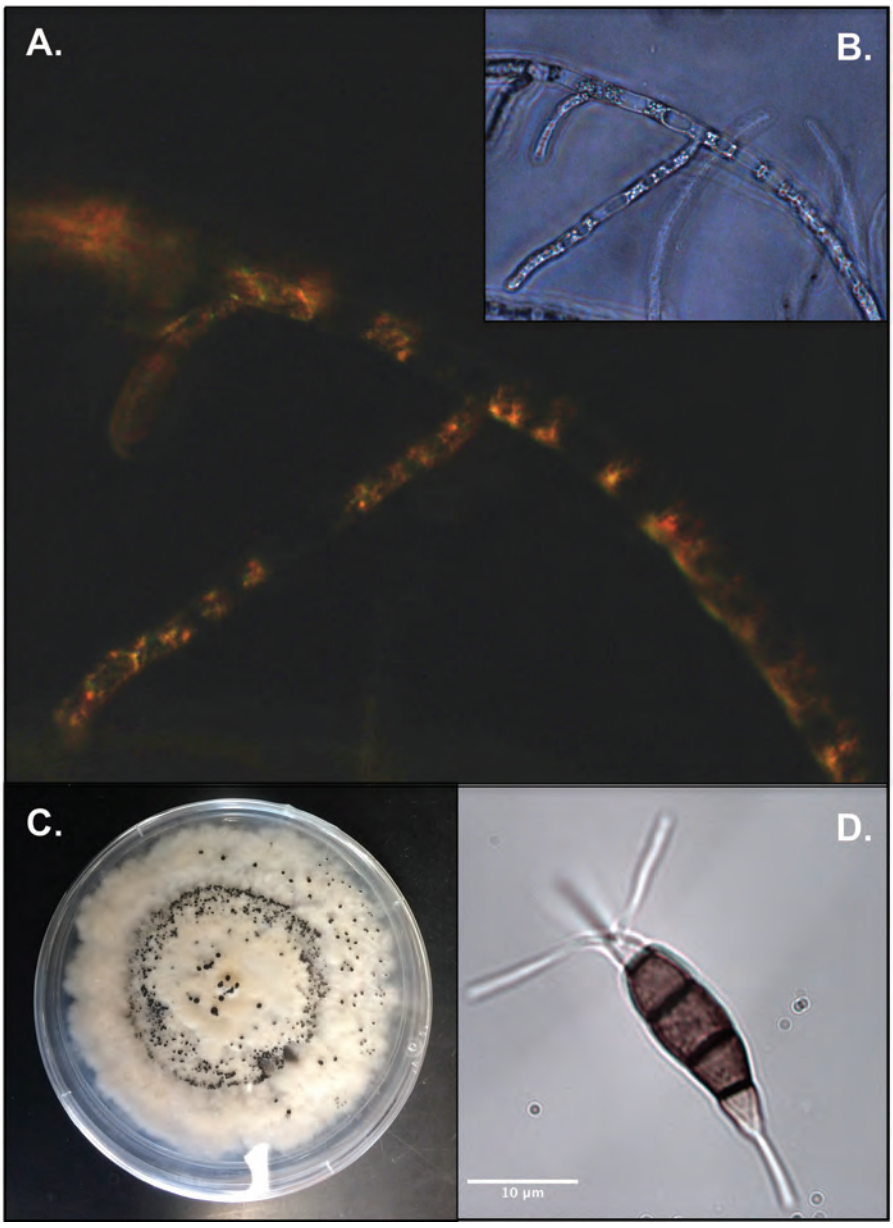


Figure 3 (**A**) Fluorescence microscopy (400×) reveals successful reintroduction of the class 3 EHB *Luteibacter* sp. 9143 with the tdTomato construct into hyphae of *Pestalotiopsis* sp. 9143. (**B**) The same image under phase contrast. (Images reprinted with modification from reference 48). (**C**) Healthy and apparently axenic colony of *Pestalotiopsis* sp. 9143 in which EHB are present but not visible without microscopy. (**D**) Conidium of *Pestalotiopsis* sp. 9143. Although EHB are widespread in the culture that produced such conidia, no conidial transmission (i.e., no vertical transmission) has been detected.

seed-associated fungus *in vitro* (19), and their genomic features (Fig. 8) (43).

Together, ca. 100 studies since 1996 have explored the biological, genomic, biochemical, evolutionary, ecological, and functional traits of EHB, painting a picture quite different than that encapsulated by the axenic approach to fungal biology (per reference 65): one of widespread, diverse, and important symbioses that frame fungal traits in cryptic ways. Emergent themes are that EHB are common in fungi, that they likely represent convergence in the general ability to live within fungi but do so in diverse ways with differing outcomes,

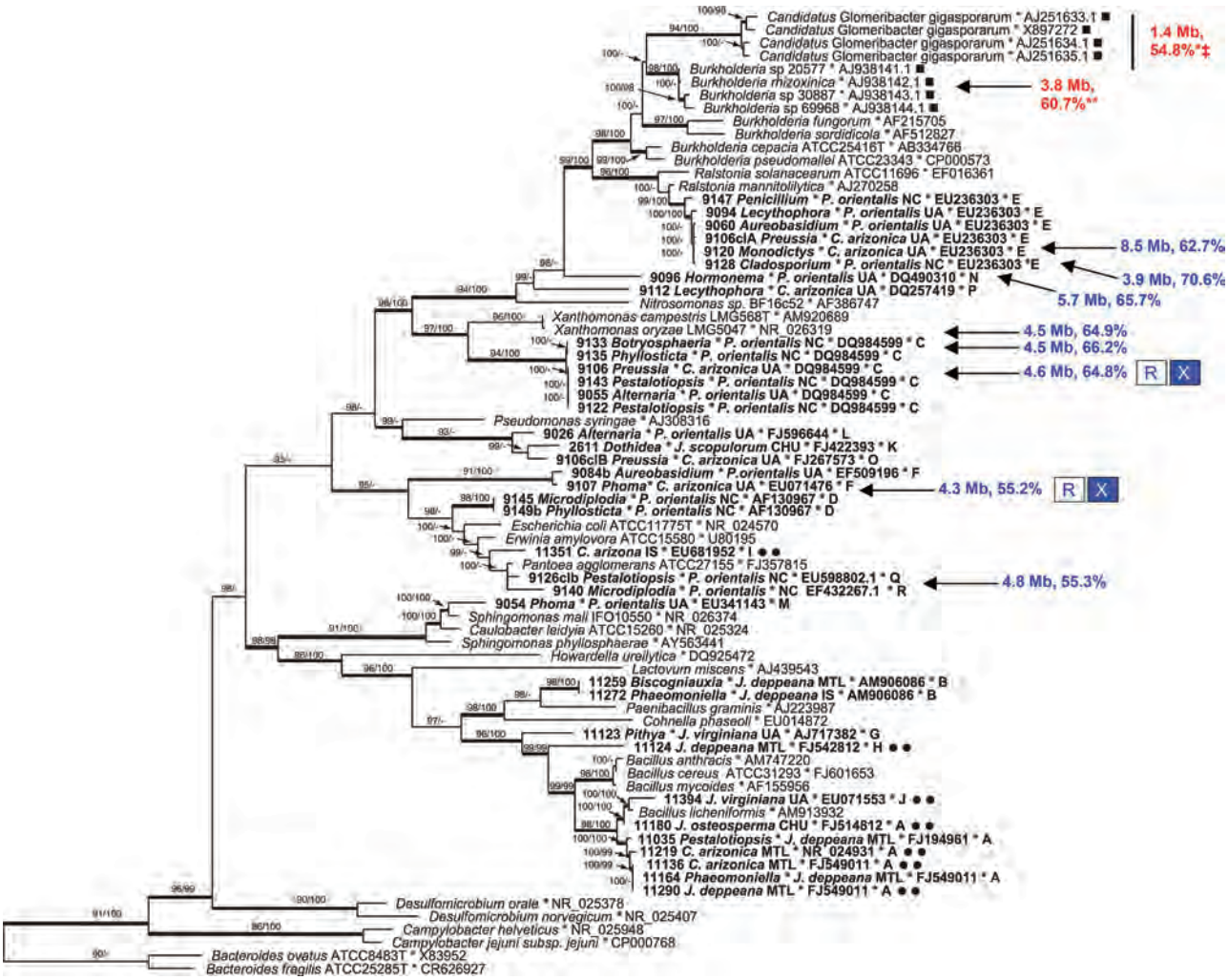


Figure 4 Phylogenetic relationships of selected class 2 EHB (black squares) associated with Mucoromycota, class 3 EHB (bold text) in the *Proteobacteria* and *Firmicutes* that associate with fungal endophytes in the Ascomycota, known bacteria (regular font), and bacterial endophytes (black circles). For fungal endophytes hosting class 3 EHB, taxon labels indicate the fungal genus, the plant species from which these fungi were obtained (*Cupressus arizonica*, *Juniperus deppeana*, *Juniperus scopulorum*, *Platycladus orientalis*), the location in which the host tree was growing (NC, North Carolina; UA, University of Arizona Campus Arboretum, Tucson, Arizona; CHU, M, and MTL, montane regions of Arizona), relevant GenBank accessions, and bacterial genotype groups (operational taxonomic units) based on 97% similarity of bacterial 16S rRNA (38). Colored labels indicate genome size and GC content for class 2 EHB (red) and class 3 EHB (blue), with the latter highlighting that members of the same 16S rRNA operational taxonomic unit can differ markedly in their genomic traits. Boxes containing R and X indicate class 3 EHB that have been reassociated with cured hosts under laboratory conditions (R) and transferred successfully and stably into novel fungal hosts (X). Labels for bacterial endophytes are similar to those of EHB in fungal endophytes but lack fungal hosts, because these bacteria occurred directly in plant tissues. Phylogenetic reconstruction from reference 38 depicts the results of a Bayesian analysis of 16S rRNA gene sequences. Branch support values indicate parsimony bootstrap values (≥70%; before slash) and Bayesian posterior probabilities (≥95%; after slash). Branches in bold indicate ≥70% neighbor-joining bootstrap values. Endohyphal *Burkholderia* listed here have since been reclassified as *Paraburkholderia* (79).

Fungal endophyte	Bacterium	Water agar 25°C	Malt agar 25°C	Water agar 36°C	Malt agar 36°C	Lignin medium	Cellulose medium	Cellulase activity
9140 - *Microdiplodia*	*Pantoea* sp.	ns	– > +	ns	– > +	nd	nd	ns
9145 - *Microdiplodia*	*Erwinia* sp.	+ > –	ns	ns	ns	ns	ns	ns
9145 - *Microdiplodia*	*Luteibacter* sp. 1	– > +	– > +	– > +	+ > –	ns	ns	+ > –
9055 - *Alternaria*	*Sphingomonas* sp.	– > +	– > +	– > +	– > +	– > +	ns	+ > –
9128 - *Cladosporium*	*Burkholderia* sp.	– > +	ns	– > +	ns	ns	ns	+ > –
9143 - *Pestalotiopsis*	*Luteibacter* sp. 2	ns	ns	+ > –	ns	ns	+ > –	+ > –

Figure 5 Context-dependence in the outcomes of interactions between class 3 EHB and foliar endophytes (rows) and meaningful phenotypic variation among symbiotic partners (columns). Cells reflect the significance and directionality of repeated-measures analyses of variance assessing growth of fungal strains containing EHB and clones that were cured of EHB by antibiotic treatment *in vitro* over 14 days on water agar (low nutrient), malt extract agar (high nutrient), lignin medium (with indulin as the sole carbon source), and cellulose medium (carboxymethylcellulose as the sole carbon source). Thermotolerance was assessed on two media at 36°C. Cellulase activity was measured as zone-of-clearing scaled by colony diameter. Orange cells indicate that the growth or cellulase activity of clones with EHB significantly exceeded that of cured clones. Blue cells indicate that the growth or cellulase activity of cured clones significantly exceeded that of clones with EHB. Gray boxes indicate no significant difference (ns) as a function of EHB status. Fungi in red differ only in the identity of their EHB, in that their fungal genotypes at the barcode locus are identical. The endohyphal *Burkholderia* listed here is now *Paraburkholderia* (79).

and that they vary reliably in suites of characteristics that can be used to place them into an operational classification. Below we define a preliminary classification for EHB before exploring in greater detail their functional significance. We then focus on one functional group to explore methods of study and research frontiers.

FUNCTIONAL CLASSIFICATION, TRAITS, AND SIGNIFICANCE OF EHB

When BLOs were first described from hyphae and spores of various Glomeromycotina, three interpretations were put forward with respect to their potential functional significance: (i) they might be obligate symbionts living in close and sustained relationships with their host fungi, (ii) they might be transient associates, repeatedly lost and reacquired by fungi, and/or (iii) they might be organelles of uncertain function and origin (68). Evidence now supports the first two interpretations for diverse EHB, albeit in different functional groups of fungi and bacteria. Here we assign a functional classification to EHB that references the phylogenetic classifications and functional traits of host fungi, genomic traits of EHB, and corresponding ecological and evolutionary traits of EHB such as transmission mode (Table 1). We identify three functional classes, here defined as class 1, class 2, and class 3 EHB.

Class 1 EHB

Class 1 EHB (Table 1) correspond to the EHB typically referred as *Mollicutes*-related endobacteria. These are consistent with the small, coccoid BLOs observed over the past half-century in arbuscular mycorrhizal fungi (41, 42, 68, 76, 87–90). These members of the phylum *Tenericutes* are closely related to various *Mycoplasma* species in the class *Mollicutes* (41, 42) and are thought to represent ancient associates of Glomeromycotina and related fungi (42, 87). Recently, a genus was erected to encompass these EHB (*Candidatus* Moeniiplasma) (91). They have not yet been isolated in culture (91).

Overall, class 1 is characterized by high intrahost genomic diversity, an apparently global distribution, and phylogenetic structure that mirrors species distinctions in their hosts (41, 42). They are considered heritable, although horizontal transmission coupled with recombination has been suggested to explain diverse intrahost populations (41, 88, 91). They have been observed in Mucoromycotina (e.g., *Endogone lactiflua* and close relatives) and Glomeromycotina (e.g., members of the Archaeosporales, Glomales, and Diversisporales [87, 90]), where they occur in the cytoplasm of fungal cells (89, 92). They typically are small (300 to 600 nm) and coccoid in appearance (89). Initially, the appearance of an electron-dense layer outside the plasma membrane led to the suggestion that they might have cell walls similar to those of Gram-positive bacteria, but this has since been refuted (41, 42, 91). They are not surrounded by host-derived membranes but instead are localized directly in the cytosol of living cells (87, 89).

The genomes of class 1 EHB are small relative to other known EHB (Table 1), but their genome sizes are consistent with those of horizontally transmitted

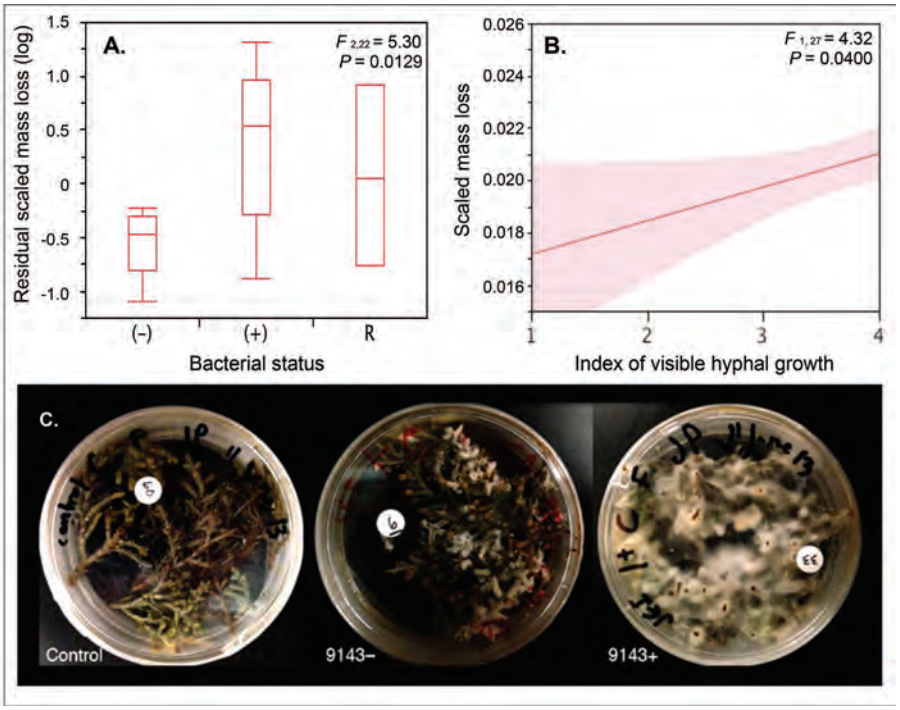


Figure 6 (**A**) Significantly more mass is lost from surface-sterilized, senescent leaves of a focal tree (*Cupressus arizonica*) following inoculation with *Pestalotiopsis* containing a class 3 EHB (*Luteibacter* sp. 9143, marked with +) versus treatment with clones of the same fungus cured of its EHB (–). Reassociation of the EHB and fungus following curing (R) led to mass loss that did not differ significantly from the original EHB-fungi association. Data reflect 14 days postinoculation in moist chambers. (**B**) Scaled mass loss is positively correlated with the quantity of visible hyphae on tissue (0 = no visible hyphae; 4 = 100% of foliage covered with fungal growth). (**C**) Hyphal coverage (white) after 14 days on surface-sterilized, presenescent foliage of *C. arizonica* in moist chambers. Controls (water only) had no visible hyphal growth. Hyphal coverage in *Pestalotiopsis* with EHB exceeded that of *Pestalotiopsis* without the EHB.

Mycoplasma (90). Genomes of *Mollicutes*-related endobacteria sequenced to date are characterized by low GC content (Table 1), low gene content, and proteins with domains involved in interactions with eukaryotic hosts, as well as genes acquired by HGT from arbuscular mycorrhizal fungi (41, 42). Genes acquired from their hosts include protein domains relevant to fungal programmed cell death, self/nonself recognition, and post-translational modification (summarized in reference 90). Their genomes also show evidence of intimate interactions with plectroviruses (*Inoviridae*, single-stranded DNA viruses affiliated with diverse *Tenericutes* [41]).

At present there is some debate regarding the functional roles of class 1 EHB. Their metabolic reliance on their fungal hosts is underscored by their limited biosynthetic capacity (summarized in reference 90). Genome analyses designed to infer function are hindered by the genetic distinctiveness of class 1 EHB, because functional annotation is lacking for >50% of genes (90). The conclusions of two contemporaneous papers differ with respect to the potential for class 1 EHB to be antagonists or mutualists with respect to their host fungi (41, 42, 88).

Class 2 EHB

In terms of host breadth, genome size, genomic traits, obligacy, and metabolic reliance on their hosts, the diverse assemblage here identified as class 2 EHB is intermediate between classes 1 and 3 (Table 1). These bacteria associate with diverse Mucoromycota and Ascomycota (Table 1). Members of class 2 demonstrate a capacity for transmission via spores as well as growth in vegetative hyphae and reproductive structures such as ascocarps (e.g., in the ectomycorrhizal fungus *Tuber borchii*) (15, 93, 94). They are best known in soilborne pathogens, seed-infecting fungi, ectomycorrhizal fungi, and the arbuscular mycorrhizae in which they were first characterized.

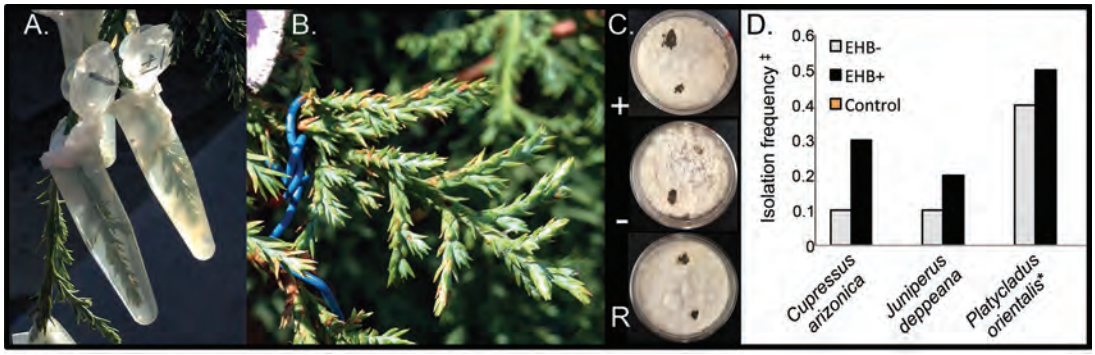


Figure 7 Class 3 EHB can modulate the establishment of endophytic fungi in healthy foliage. (**A**) Surface-sterilized foliage of the focal tree species, *Cupressus arizonica*, was immersed in a suspension containing axenic hyphae, a suspension containing hyphae with the EHB, or water only. Tubes were left in place for 24 h and then removed. (**B**) Inoculated branches were marked with colored wire according to treatment. After 2 weeks, tissue was surface-sterilized and evaluated for endophytes. (**C**) The foliar endophyte was reisolated from surface-sterilized, inoculated plant tissue (+, endophyte with EHB; –, axenic endophyte; R, reassociated EHB and endophyte). These strains do not differ in growth on standard media under laboratory conditions. (**D**) Successful inoculation by the axenic endophyte (EHB–, gray) and especially by the endophyte with EHB (EHB+, black); *y* axis shows percentage of 2-mm tissue segments from which the fungus was reisolated. The endophyte was never observed in untreated foliage (control, orange). *Host species from which focal symbiotic pair was first isolated.

Class 2 EHB have moderately sized genomes (ca. 1.4 to 3.8 Mb), moderate GC content (ca. 46 to 60%), a predominance (although with some exceptions) of vertical transmission, limited culturability or a high degree of fastidiousness, and in many cases, occurrence in fungal cells within membranes of host origin (Table 1) (15, 89). Some EHB in this class have yet to be isolated in culture (see 19, 93–95), but some can be cultivated outside of their hosts under laboratory conditions (93). Homogenizing mycelia and using genomics to predict substrate use or optimize growth conditions may greatly increase the success with which EHB in this functional group can be isolated in culture (96).

As presently defined (Table 1), class 2 EHB include members of the *Burkholderiaceae* (*Betaproteobacteria*) such as "*Candidatus* Glomeribacter gigasporum" in various Glomeromycotina (95, 97, 98), *Paraburkholderia rhizoxinica* and *Paraburkholderia endofungorum* in *R. microsporus* (Mucoromycotina [99–101]), and *Mycoavidus cysteinexigens* in *Mortierella elongata* (Mortierellomycotina [44, 96]). Also included here are intriguing examples of vertically transmitted and/or apparently unculturable associates of Dikarya, including the *Cytophaga-Flexibacter-Bacteroides* clade in ectomycorrhizal *T. borchii* (Ascomycota) (94, 102) and *Chitinophaga* sp. (Bacteroidetes) in a seed-associated *Fusarium keratoplasticum* (Ascomycota) (19, 49).

Such class 2 EHB illustrate the range of genomic and ecological traits that characterize this diverse functional group. Here we explore the four best known of these: "*Candidatus* Glomeribacter gigasporum," *P. rhizoxinica*, *M. cysteinexigens*, and *Chitinophaga* sp.

"*Candidatus* Glomeribacter gigasporum" (*Burkholderiaceae*) is an EHB affiliated with primarily Glomeromycotina such as *G. margarita*, *Scutellospora persica*, and *Scutellospora castanea* (97). It is vertically transmitted through spores and has not been grown in culture outside of its hosts (103), although it can be separated from host mycelium and maintained for several weeks under laboratory conditions (98). Consistent with vertical transmission and a high degree of metabolic dependence on its host, it has a relatively small genome (1.4 Mb with one plasmid [98]; 1.72 Mb with three plasmids [93]) and limited capacity for carbon catabolism, gluconeogenesis, phosphate transport, and the synthesis of essential amino acids. In addition to synthesizing diverse metabolites, including antibiotics, vitamin B_{12}, and molecules relevant to resistance against toxins (93), it influences the protein expression and lipid profiles of its host fungus, as well as spore morphology and hyphal growth in presymbiotic phases (104, 105). Like a related member of class 3 EHB (*Paraburkholderia* sp. 9120; see Fig. 8 and discussion below), it expresses type II and type III secretion systems (43, 93).

Figure 8 Genomic traits of class 3 EHB associated with foliar endophytes in the Ascomycota (blue font) compared with nonendohyphal relatives (black) and a model class 2 EHB (red), showing the bacterial habitat, genome size, gene count, and results of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway searches to identify bacterial pathways involved in signaling between bacteria and eukaryotes (43). Boxes along the *x* axis indicate KEGG pathway identifiers (top) for constituent genes for each bacterial secretion system. Colored boxes indicate that at least one gene within the genome is present and classified according to that specific KEGG identifier. Numbers inside the colored boxes denote that more than one gene within that genome is classified according to that KEGG identifier. Boxes for EHB bacteria described first in reference 43 are shown in blue. Green boxes highlight a species that interacts with fungi but does not appear to occur endohyphally. Endohyphal *Burkholderia* listed here are now reclassified as *Paraburkholderia* (79).

Table 1 Functional classification of endohyphal bacteria into three operational classes based on host information, bacterial phylogeny, genomic traits, and associated traits relevant to bacteria/fungi interactions and ecology (references listed in text)

Class	Equivalency or example[a]	Fungal clade	Bacterial clade	Genome size (Mb)	GC content (%)	Transmission	Obligacy of bacterial association	Metabolic reliance on host	Localization	Cell wall	Proposed interaction with host
Class 1	*Mollicutes*-related endobacteria (MREs)*	Mucoromycota (Glomeromycotina, Mucoromycotina)	*Tenericutes*	0.7–1.2	32.0–34.3	Vertical	Obligate	High	Cytoplasm	No	Debated and presently unknown
Class 2	*Burkholderia* (*Paraburkholderia*) *rhizoxinica*‡ and *Paraburkholderia endofungorum*‡, "*Candidatus* Glomeribacter gigasporum"‡, *Chitinophaga* sp. EHB01‡, *Cytophaga-Flexibacter-Bacteroides*‡	Mucoromycota, soilborne Ascomycota	*Proteobacteria*, *Bacteroidetes*	1.4–3.8	46.1–60.7	Vertical but potentially horizontal in some species	Obligate to facultative	Moderate to high	Cytoplasm; some surrounded by membrane of fungal origin	Yes	Generally beneficial but can be context-dependent
Class 3	*Luteibacter* sp. 9143‡, *Erwinia* sp. 9145‡, *Pantoea* sp. 9140‡, *Pantoea sp*. 9133‡, *Massilia* sp. 9096‡, *Rhizobium* sp. 9140‡, *Curtobacterium* sp. 9128‡, *Paenibacillus* sp.‡, *Rhizobium radiobacter*‡	Diverse Ascomycota, Basidiomycota	*Proteobacteria*, *Firmicutes*, others	3.9–8.5	55.2–70.6	Horizontal	Facultative	Moderate	Cytoplasm; some surrounded by membrane of fungal origin	Yes	Mixed; highly context-dependent

[a]*, equivalency; ‡, example.

Ghignone et al. provide a model for tripartite interactions among "*Candidatus* Glomeribacter gigasporum," its fungal host, and the host plant with which these symbionts interact, highlighting energy fluxes in living cells, metabolic exchange, and the occurrence of the EHB in protein-rich vacuoles (93). This species is a model for studies of plant-arbuscular mycorrhizal-EHB associations.

Paraburkholderia rhizoxinica (formerly *Burkholderia rhizoxinica*) is affiliated with *R. microsporus* (Mucoromycotina), in which it is responsible for producing the toxins that have been identified as causal in rice seedling blight disease (14, 52). These include rhizoxin, a potent antimitotic agent, and rhizonin, a hepatotoxic cyclopeptide, both of which were previously thought to be mycotoxins produced by *R. microsporus* (52, 99, 101). Interestingly, the fungal host requires *P. rhizoxinica* for sporangium and spore development, tightly linking EHB with fungal reproduction and fitness (100). The same authors developed techniques for labeling and artificially introducing the bacterium into fungal structures, thus establishing a highly informative experimental system for studying EHB-fungi interactions (100). By studying resistance of fungi to the antimitotic properties of rhizoxin, Schmitt et al. suggested that the stable affiliation of *P. rhizoxinica* with *R. microsporus* represents a transition from parasitism to mutualism (106). Consistent with its close association with its fungal host, *P. rhizoxinica* has a 3.75-Mb genome with primary metabolism oriented to the acquisition and use of fungal metabolic products (107). Like many other EHB, and many non-EHB in the *Burkholderiaceae*, it is characterized by the presence of multiple plasmids in addition to its own chromosome. The biosynthetic gene cluster relevant to rhizoxin is located on the bacterial chromosome itself (107). Although it is transmitted via spores, evidence from a global population study suggests that past horizontal transmission events have shaped the genomic architecture of this species (47). *P. rhizoxinica* can be grown with limited success in culture under laboratory conditions (47, 52, 107). This EHB can actively invade fungal hyphae in part through use of a type II secretion system, which releases chitinolytic enzymes (107, 108). Strikingly, incursion into fungal cells occurs via softening of the fungal cell wall and leaves little detectable trace (108). In pure culture the EHB is motile, occurring in pairs or at times in clusters as short or coccoid rods ca. 1.2 to 2 μm long and 0.6 to 1.2 μm in diameter (107). This EHB was the first to be characterized as markedly altering the phenotype of fungi with respect to plant pathogenicity and toxin production (14), and it is a model system for genomics and the study of plant pathogen-EHB interactions. A close relative, *Paraburkholderia endofungorum*, was described in 2007 and shares many traits with *P. rhizoxinica* (109).

Mycoavidus cysteinexigens (*Burkholderiaceae*) is an EHB associated with the soil-inhabiting fungus *Mortierella elongata* (Mortierellomycotina) (44, 63, 110). This bacterium has been studied primarily via metagenomics (44) and genome sequencing of the bacterial fraction released from homogenized or macerated mycelium (110). Its 2.6-Mb genome reflects reduced function and considerable reliance on the fungal host (111). Detection of a potential cysteine dependency from genomic analyses led to its successful isolation (110), and treatment with antibiotics has revealed that it plays a major role in modulating host metabolism (44). The association between *Mortierella* and *Mycoavidus*, like that between other EHB and Glomeromycotina, is thought to be ancient, spanning in this case an estimated 350 million years (44).

Unlike the above examples, which encompass EHB in the *Burkholderiaceae* that occur in fungi in the Mucoromycota, *Chitinophaga* sp. EHB01 (*Bacteroidetes*) is affiliated with a member of the Ascomycota. It was isolated originally from *F. keratoplasticum* PS0362A, which in turn was isolated from a seed of a tropical tree (*Cecropia insignis*) after exposure to soil for several weeks in a lowland forest understory (19, 49). The genome of this EHB has not yet been sequenced, but the relevance of the *Chitinophaga-Fusarium* association has been evaluated using phenotypic microarrays. Since their development, phenotypic microarrays have been extended from evaluations with bacteria only to studies of yeasts and filamentous fungi. Shaffer et al. used phenotypic microarrays to compare growth of *F. keratoplasticum* with and without *Chitinophaga* on diverse carbon substrates (19). The fungus grew readily on standard growth media and on diverse substrates in the absence of the EHB, and on standard media, colony diameter and spore production were similar regardless of the presence of the EHB. However, global substrate use (i.e., growth across all substrates considered simultaneously) differed markedly between clones as a function of *Chitinophaga* infection. In particular, the axenic fungus grew to a higher density over the course of the experiment on 5 (of 95) carbon substrates (1 monosaccharide, D-arabinose; 2 stereoisomeric forms of one carboxylic acid, D- and L-malic acid; and 1 amide, succinamic acid). However, with its EHB, the fungus grew to a higher density on 59 of 95 substrates, including >75% of sugar-based substrates and most amino- and carboxylic acids and their derivatives (19).

Thus, *Chitinophaga* EHB01 is associated with increased breadth of substrate use and enhanced growth by its host on many substrates. Moreover, its improvement of fungal growth on substrates relevant to plant- and seed-fungus interactions is intriguing from an ecological standpoint. Much as genomic insights can be used to infer phenotypic traits, here phenotypic observations provide the basis for predictive genomics, currently a target for future work. Although vertical transmission of *Chitinophaga* has not yet been observed, we anticipate that challenges associated with isolating it in culture will correspond to a relatively small genome that is tightly integrated with host functional traits. This argues for its placement in class 2, much like the *Cytophaga-Flexibacter-Bacteroides* symbiont of the ectomycorrhizal ascomycete *T. borchii* (102). However, genomic data are needed for these strains, which stand out from other class 2 EHB by occurring in septate fungi within the Dikarya, rather than in the coenocytic Mucoromycota described above. These Ascomycota-associated *Bacteroidetes* may prove to be a unique functional group or may be moved to class 3 should cultivation be achieved and transmission by spores not be detected.

Class 3 EHB

Class 3 EHB are a diverse, nonmonophyletic assemblage of bacteria characterized primarily by horizontal transmission, partially free-living or otherwise non-endohyphal lifestyles, high phylogenetic diversity, and occurrence in the living hyphae of Dikarya (11, 17, 19, 38, 43, 48, 49, 85, 112). As a group these EHB represent the second scenario outlined by MacDonald et al.—that EHB may be transient associates of their hosts, with the capacity to be gained and lost repeatedly (68; see also 38, 48). Such gains and losses are easily detectable in the laboratory and speak to a facultative lifestyle in nature (17, 38, 48, 49).

Class 3 EHB as defined here occur in hyphae of Ascomycota and Basidiomycota affiliated with soil and above-ground plant parts. Hosts include ectomycorrhizal *Laccaria* (*Basidiomycota* [84]), growth-promoting root endophytes such as *P. indica* (*Basidiomycota* [85]), and diverse Pezizomycotina that occur in leaves and seeds of plants from tropical to arctic settings (38, 49). Together, class 3 EHB represent at least the phyla *Proteobacteria* (including *Alpha-*, *Gamma-*, and *Betaproteobacteria*; Fig. 4) and *Firmicutes* (e.g., *Paenibacillus* in *Laccaria* [112]; see also reference 38 for a report of *Paenibacillus* in *Phaeomoniella* and *Biscogniauxia* strains that occur as foliar endophytes).

The bulk of the diversity in this functional group appears to reside in the Proteobacteria, represented by genera such as *Paraburkholderia* (with large genome sizes compared to congeneric bacteria occurring as class 2 EHB [Fig. 4]); *Rhizobium* and *Acinetobacter*, associated with Sebacinales; and *Luteibacter*, *Pantoea/Erwinia*, *Sphingomonas*, and others (Fig. 4; Table 1). Generally, these bacteria are closely related to free-living or other non-EHB lineages (38, 43). They can be visualized using LIVE/DEAD staining and fluorescence *in situ* hybridization (Fig. 2). In some cases the number of bacterial cells per fungal cell can be quite low (e.g., 2 to 20 per fungal cell [113]). The few cases in which bacterial DNA has been quantified with respect to fungal genomic DNA reveal concomitantly low amounts of bacterial genomic material (e.g., 0.035 ng of bacterial DNA:100 ng of fungal DNA [114]).

Class 3 EHB typically can be cultured outside of their hosts, reintroduced to their axenic hosts, and moved into novel fungal strains (Fig. 3) (17), presenting a tractable set of experimental systems for tracking the relevance of EHB in modulating fungal phenotypes (11). At least some species are encapsulated in the cytoplasm of Dikarya by membranes apparently of host origin (M. Hoffman, personal communication). Individual fungi can harbor more than one class 3 EHB in different parts of the same mycelium (49), with EHB transformation allowing visualization of single- and multi-EHB infections (see Fig. 3).

Class 3 EHB appear not to have undergone significant reductions in genome size or content relative to their close relatives with other lifestyles. The genomes of these EHB generally are larger than those of class 1 and class 2 EHB (Table 1; Fig. 4), with higher GC content and a prevalence of genomic traits consistent with life phases outside of fungal hyphae (43). Their genome sizes range from ca. 3.9 to 8.5 Mb, with GC content exceeding 70% in some strains (Table 1; Fig. 8). Most have circular chromosomes and varying numbers of plasmids, but *R. radiobacter* has both a circular chromosome and a linear chromosome (together accounting for 4.9 Mb) as well as one plasmid (0.21 Mb) (114). GC content (ca. 59.3 to 59.4% in the bacterial chromosome) is similar to that of many other class 3 EHB (Table 1). Like other class 3 EHB, *R. radiobacter* is closely related to bacteria that do not occur as EHB (in this case, to plant-pathogenic bacteria [114]).

Preliminary evidence indicates profound effects of individual class 3 EHB on their fungal hosts with respect to altering phytohormone production, thermotolerance, substrate use, and the production of plant cell wall-degrading enzymes (Fig. 5) (see also references

11, 17). For example, *Luteibacter* sp. 9143 markedly increases production of indole-3-acetic acid in a foliar endophyte (*Pestalotiopsis* sp. 9143) (11). This increase in hormone production enhanced growth of seedlings treated with culture filtrates (11). However, few general rules apply with regard to the effects of these diverse bacteria on their hosts. For example, two closely related strains of endohyphal *Luteibacter* are associated with different phenotypic responses in different fungi (Fig. 5). Similarly, the same fungus differs markedly in its phenotype with respect to substrate use and thermotolerance when infected by different class 3 EHB (Fig. 5).

Overall, the emergent picture is one of facultative associations with context-dependent outcomes, which together translate to impacting plant-fungus symbioses in profound but as yet unpredictable ways. In this way, class 3 EHB in fungi such as endophytes resemble the facultatively symbiotic fungi they frequently inhabit, whose benefits for hosts are often context-dependent. Even so, class 3 EHB may play important roles in fungal niche differentiation, substrate use, and ultimately, ecological modes in nature: in the partnerships studied so far, certain EHB are associated with enhanced saprotrophy by some fungal endophytes (e.g., *Pestalotiopsis* sp. 9143), concomitant with enhancement of growth on cellulose-containing media and the production of cellulolytic enzymes (Fig. 5). In other cases symbiotic establishment with host plants can be influenced by class 3 EHB, in the context of both leaves (Fig. 6) and roots (113). Increasingly, it appears that EHB are important in the ecological diversification of fungi in the short term, potentially with lasting evolutionary consequences that may speak to the hyperdiversity of endophytic fungi and related functional groups of Dikarya (e.g., 115, 116).

Concomitant with their ecological and evolutionary diversity, class 3 EHB exhibit diverse genomic features relevant to bacteria-fungi interactions (Fig. 8). Closely related EHB often have similar genomic architecture with respect to secretion systems and enzyme-encoding genes, and often they resemble the genomic structure of their close relatives that occur in other ecological settings (Fig. 8). The diverse class 3 EHB for which genomes are sequenced demonstrate convergence on the endohyphal lifestyle, but apparently via different evolutionary pathways and with different mechanisms for communication with fungal cells (Fig. 8).

FRONTIERS FOR STUDYING EHB

The study of EHB was initiated when observations of fungal structures by microscopy revealed the presence of unexpected structures resembling bacteria in the cytosol (53, 66, 68, 76). Since that time inquiries into the existence, identity, and function of EHB have followed a trajectory that includes imaging; curing and, where possible, reinfection; phylogenetic characterization; and most recently, genomics and metagenomics that have in some cases facilitated culturing and in other cases illuminated the evolutionary history of uncultivable species (41, 42, 88). In the near future metatranscriptomes will be exciting for understanding gene expression, particularly in class 2 and class 3 EHB whose relationships with fungi can be manipulated. Variation in the outcomes of associations between class 3 EHB and different fungi (Fig. 5) sets up an especially compelling area of inquiry in which metatranscriptomic perspectives will be enlightening. Those fungus-EHB pairs that can be cured and reassociated, or that can be introduced easily into other fungi, might be promising targets for such work (see Fig. 3, Fig. 4, Fig. 5).

In the interim, current and traditional methods of evaluating fungi for EHB continue to be fruitful. The prevalence of class 3 EHB in the species-rich Dikarya argues for screening existing fungal collections and new isolates from diverse phylogenetic and functional groups to understand the scope and distribution of EHB symbioses. At the moment, most known EHB are in plant-associated fungi; EHB (class 2) have been detected in clinical isolates of *R. microsporus*, but the bacteria are not required for zygomycoses (47). Given the importance of such EHB as producers of toxins, however, an important area for future research relevant to human health certainly lies in food safety (e.g., in the context of *Paraburkholderia* affiliated with *Rhizopus* strains used for tempeh production; see references 47, 117).

In the earliest studies that detected EHB, transmission electron microscopy was key for diagnosing the presence of BLOs in hyphae of arbuscular mycorrhizal fungi (53). A decrease in the training of young mycologists in transmission electron microscopy and related methods (including ultrastructural analysis and other imaging approaches such as scanning electron microscopy) may limit visualizations that so richly complement -omics studies of EHB. In the case of class 3 EHB, resynthesis conditions have been published for the association between *Pestalotiopsis* and *Luteibacter*, outlining an approach for manipulating EHB and their partners that could be translated to microscopy and linked directly to genomics/metatranscriptomics analyses (48). In seeking to link structures with genes and gene expression, mycologists may benefit in some cases from collaborations: a survey of the literature reveals

that few papers on EHB have been published in mycological journals, suggesting untapped potential to link fungal biology with the work of microbial and genomic scientists outside the traditional scope of mycology.

At the end of the 20th century, intracellular *Paraburkholderia* (class 2) were described in the cytoplasm of Glomeromycotina through the combined approaches of PCR and fluorescence microscopy (e.g., 46). Staining spores of *G. margarita* with LIVE/DEAD (46) confirmed the viability of both the fungal and bacterial symbionts, a much-needed indication of symbiosis that should be established clearly when new EHB-fungi associations are described (see 15). Similarly, Salvioli et al. used SYTO BC dye, a nucleic acid stain component, in conjunction with confocal microscopy to detect "*Candidatus* Glomeribacter gigasporum" in spores of *G. margarita* (13). Such methods persist in more recent studies as effective approaches for validating the presence and placement of living bacteria in diverse fungi (38, 48, 49). However, methods such as LIVE/DEAD can lead to uncertain interpretations: fluorescence by ectohyphal bacteria and fungal nuclei and mitochondria cannot always be distinguished easily from EHB. In these cases, combining molecular analysis and more specific methods such as fluorescence *in situ* hybridization can be helpful. For example, Hoffman and Arnold used phylogenetic approaches based on 16S rRNA in conjunction with a TAMRA fluorophore to visualize and unambiguously confirm the identity of class 3 EHB (Fig. 2) (38).

Here, too, transformation of EHB can be of immense help in visualizing bacteria within fungal cells. For example, Partida-Martinez et al. used green fluorescent protein labeling to track vertical transmission of class 2 EHB (100). Similarly, Arendt et al. imaged tdTomato-tagged *Luteibacter* sp. 9143 in hyphae of its host (48). In the latter study, the *Luteibacter* strain was manipulated with transposon mutagenesis (Fig. 3) (48). In such cases, EHB that can be grown axenically, be manipulated, and be reintroduced to hosts can be especially tractable.

In general, class 3 EHB can be isolated from fungal hyphae after prolonged immersion in water, after heat treatment, or after macerating or otherwise damaging fungal mycelia (an approach that also has been useful in segregating some class 2 EHB for molecular analysis [96]). The emergence of EHB in culture, particularly from vouchers of fungi such as endophytes stored in sterile water, sometimes can be mistaken for contamination if their presence is unexpected (38). Occasionally, EHB may overcome fungi vouchered in water, making it impossible to regrow stored fungal strains. In such cases, fungaria may do well to voucher dual cultures: those grown on antibiotic-containing media and those grown on standard media (38). If fungi are originally isolated from the environment using antibiotic-containing media, EHB may be removed before downstream studies occur, and in such cases vital aspects of fungal traits—shaped by their EHB—might be overlooked. Because some fungi may harbor more than one EHB strain concurrently (49), future work should incorporate next-generation sequencing methods to evaluate the diversity and composition of EHB communities in individual mycelia.

More generally, a wealth of new or newly applied methods provides a compelling opportunity to address questions that are fundamental to understanding the traits and importance of EHB. Here we explore these with respect to molecular approaches, imaging, and questions of interdomain communication. In the following discussion we focus primarily on EHB that can be isolated and grown in culture, which are tractable for studying the initial phases of bacteria-fungi interactions, their chemical currency, their genetic and phenotypic responses to one another, the timeline of their associations, and—as demonstrated elegantly by Moebius et al. (108)—the dynamics of bacterial colonization into fungal hyphae. In this context, approaches used to study bacteria-fungi interactions in nonendofungal systems can shed light on principles of interdomain associations and their outcomes in the context of EHB symbioses. We explore several such avenues below, especially with regard to frontiers in the study of class 3 EHB, a ubiquitous group of symbionts with horizontal transmission that appears widespread in the most species-rich fungal clades.

Frontiers in Transcriptomics and Genomics

As the cost of high-throughput sequencing drops and more genomic data become available, opportunities will increase to understand the genetic underpinnings that shape bacteria-fungi interactions at stages ranging from first encounters to symbiont-driven modulation of fungal traits. In particular, transcriptomics tools have become increasingly important for understanding how genes are regulated during biotic interactions. For example, by comparing transcriptomes of the fungus *Aspergillus niger* and the bacterium *Bacillus subtilis* during coculture and axenically, Benoit et al. observed obvious metabolic changes upon attachment of the bacteria to fungal hyphae (12). Both *B. subtilis* and *A. niger* decreased their production of antifungal and antibacterial molecules, respectively. The majority of the genes downregulated during these *in vitro* inter-

actions were related to detoxification or secondary metabolite production and carbon metabolism, suggesting that an active physiological adaptation to the partner organism occurred in culture (12). Such dynamism may underscore facultative associations between class 3 EHB and their fungal hosts.

Similarly, Mela et al. used a dual transcriptomics approach to explore bacterial genes involved in mycophagy by *Collimonas fungivorans* and the fungal response to this attack (118). Their findings indicated a complex interaction involving antibiosis and nutrient competition in both organisms. On the bacterial side, chitinases and secondary metabolites played an important role. The fungal response was dominated by differential expression of genes related to cell wall and lipid degradation, nitrogen deficiency, and cell defense. Future studies could evaluate whether fungi exhibit similar responses when exposed to their native EHB (as opposed to EHB from other fungi) or when exposed to other bacteria that are closely related to EHB yet have other lifestyles (see 17).

Although there are commonalities in the fungal response to antagonism in both of these studies, common ground also exists between positive and negative interactions. A dual-transcriptomics microarray study of the interaction between *L. bicolor* and three bacteria (a helper strain, a commensal strain, and an antagonistic strain) revealed negative correlations in transcripts involved in primary metabolism between antagonistic and nonantagonistic interactions (119, 120). However, the fungus had a unique response to each bacterial strain. Although each bacterial strain responded differently to the fungus, all three bacteria demonstrated differences in the expression of genes involved with cell wall modification (119, 120). The relatively small size of microbial genomes and differences in mRNA processing between prokaryotes and eukaryotes make bacteria-fungi interactions ideal for transcriptome studies, with the potential to explain how EHB modulate the phenotypes of their fungal hosts in real time.

More broadly, the accumulation of genome-scale data from diverse EHB over the past 2 decades promises to accelerate as these associates of fungi, and their relevance, become better known. Comparative genomics for densely sampled clades that contain EHB has the potential to identify hypotheses regarding mechanisms of interactions between fungi and their endosymbionts. Variation in the presence of various protein secretion systems (e.g., Fig. 8) suggests that as a whole, EHB may communicate and interact with their fungal hosts in highly diverse ways. Chitinase has proved especially important for facilitating bacterial entry into living fungal hyphae (108), but it is striking that genomes of diverse EHB do not necessarily contain genes for chitinase production (Fig. 8) (43). Studies regarding alternative methods of entry into fungal hyphae merit further attention.

One related area of inquiry that also merits exploration is that of HGT between EHB and their fungal hosts. Illustrated clearly in the case of class 1 EHB (41), HGT contributes greatly to bacterial genome evolution in general (121) and has been detected, albeit at a lower frequency, in diverse fungi (122). Several genomic studies have suggested HGT between bacteria and fungi (123, 124), a process that could be accelerated or increased in frequency when fungi host EHB. We anticipate that HGT may be especially common in EHB with type IV secretion systems similar to that of *Agrobacterium*, which have been used to successfully transform fungi *in vitro* (125). Genes encoding for type IV secretion systems have been observed among diverse EHB in class 2 (e.g., plasmid-encoded in *P. rhizoxinica* [107]) and class 3 (Fig. 8) (43).

Frontiers in Metabolomics

Many new tools for studying signaling molecules relevant to bacteria-fungi interactions are increasingly available and can be applied to the study of EHB. One area of interest lies in the production and relevance of volatile organic compounds (VOCs) (see "Frontiers in Interdomain Communication," below). For example, recent advances in mass spectrometry (MS) acquisition and analysis have helped to elucidate VOCs used as novel communication signals of soilborne bacteria and fungi. Solid-phase microextraction devices are small filaments that can be inserted into the airspace of interest to adsorb VOCs and then be analyzed directly in gas chromatography MS. These devices have been used with cocultures as well as directly on substrates to identify compounds mediating intermicrobial interactions (126–128). Profiling of VOCs also is being advanced through tools such as the electronic nose, or e-nose, which does not provide specific identities of compounds in sampled air but provides entire profiles that allow researchers to make global comparisons between samples or to detect specific microbial profiles in the environment (129–132). To our knowledge, this method has not been used to examine specific bacteria-fungi interactions per se, though it has been used to profile the progression of truffle aromas over time (129), which are thought to be attributable to the fungus as well as its bacterial associates (133).

In turn, diffusible interactions are a related area of interest for exploring presymbiotic phases of EHB-

fungi interactions. Plating colonies in opposition, then extracting and assaying metabolites to examine outcomes (i.e., antibiosis, growth stimulation, chemotaxis) has been the "gold standard" in this area for some time. However, many recently developed techniques are extraction-free, which helps reduce inherent bias of extraction methods and gives a more complete picture of the molecular interplay of microbial interactions. Imaging MS is an especially promising platform, because it allows researchers not only to determine what compounds are in a sample, but also to map the location of those compounds to specific locations in coculture (134). One such method, matrix-assisted laser desorption imaging MS (MALDI-MS), is an extraction-free method that can assess the mass spectral patterns of interacting microbes on solid media. Matrix-assisted laser desorption imaging MS was used to identify ralsolamycin, the interdomain communication compound that induces formation of fungal chlamydospores (135), and to map metabolic interactions and conversion of bacterial phenazines by *Aspergillus fumigatus* (136). Finally, nanospray desorption electrospray ionization MS provides the ability to analyze metabolites directly from living environmental samples at the microscale (137, 138). When these techniques are combined with metabolic networking platforms, metabolic exchanges can be identified (139) that could inform presymbiotic and early interactions between EHB and their fungal hosts.

Frontiers in Imaging

Electron microscopy has a long and fruitful history with regard to illuminating how diverse bacteria interact with and occur in fungal hyphae (e.g., 68, 108). For example, Levy et al. used electron microscopy to explore colonization of *Gigaspora decipiens* spores and hyphae by a *Paraburkholderia* sp. (class 2 EHB) (140). Their work revealed previously undescribed fibrillar structures that were used to adhere to the hyphal surface. In parallel, improved spatial resolution has made confocal microscopy similarly indispensable for exploring close associations of bacteria and fungi, especially in conjunction with fluorescence *in situ* hybridization and related approaches (see 141). In both class 2 and class 3 EHB, interactions between bacteria and fungi can be temporally dynamic, such that monitoring these interactions as they progress is desirable (see 108). While traditional time-course studies have set the groundwork for understanding general processes in bacteria-fungi interactions (12), new tools can allow continuous, nondestructive monitoring of interactions through time. Stanley et al. recently used novel microfluidic devices to monitor interactions continuously between *Coprinus cinerea* and *B. subtilis*, showing dynamics in attachment, growth rates, and cell death resulting from these interactions (142). Such approaches provide an exciting new avenue for exploring microbial interactions over time at a microscopic level, including the dynamics of colonization by EHB, potential cell-sorting or selection of symbionts by fungi, and the abundance of colonists under particular conditions.

Frontiers in Interdomain Communication

Directed signaling between bacteria and fungi in environments such as soils often relies on VOCs (143). These compounds are generally lipophilic and have a low molecular weight, high vapor pressure, and low boiling point, which facilitates their movement through substrates via vapor diffusion and advection (i.e., matter transfer via fluid). Recent work demonstrates that VOCs are produced frequently by bacterial symbionts of plant-associated and soilborne fungi, with effects on fungal morphology, growth, and persistence in the environment (144). Such effects can be positive or negative and have strong impacts, especially at the spore germination and mycelial growth phases of fungal life cycles (145, 146).

In turn, some bacteria and fungi participate in two-way signaling. When the bacterium *Ralstonia solanacearum* was cocultured with a plant-pathogenic strain of *Aspergillus flavus*, conidiation by *A. flavus* decreased and aflatoxin production increased (127). At the same time, *R. solanacearum* showed decreased growth, decreased melanin production, and extracellular polysaccharide production (127). Sampling of the airspace of these cocultures and axenic cultures revealed VOC profiles that could influence autoinduction systems. We anticipate that further exploration of microbial signaling will show that VOCs contribute to subtler shifts in physiology than those described to date, which is potentially important in the early phases of communication between EHB and their fungal hosts.

As the above studies suggest, fungi are likely active participants in establishing interactions with EHB and, like many hosts that harbor microbial symbionts, play some role in filtering colonization by microorganisms. One particularly exciting area for future work with respect to such close interdomain interactions might focus on lectins. These proteins that react with sugars represent one of the few examples in which fungal activity is known to be influential in the early stages of interactions with bacteria. Lectins are widespread in fungi. They can mediate interactions between bacterial

and fungal pathogens and their hosts, including both plants and animals (147). Although reported primarily from Basidiomycota (148), lectins with relevance to bacteria-fungi interactions are known in some Ascomycota. For example, Díaz et al. showed that a glycosylated arginase acting as a lectin from the lichen-forming fungus *Peltigera canina* was involved in recruitment and attachment of cyanobacterial cells to the mycobiont (149). Given that both fungal and bacterial cell walls are extensively decorated with diverse carbohydrate moieties, lectins seem to be a reasonable area of focus for exploring the early signaling, chemical interactions, and attachment relevant to the establishment of EHB.

Similarly, oxalic acid is produced by members of diverse fungal phyla (150). Oxalic acid is continuously excreted from hyphal tips, sometimes in the form of oxalate crystals. Mycophagous *Collimonas* uses oxalic acid to locate hyphal tips (150). Bacterial motility experiments demonstrated that *Collimonas* can recognize and engage in directed growth based on concentrations of free oxalic acid (150). It is plausible that this and similar signaling molecules may be important in the initiation of EHB symbioses.

More generally, such observations speak to an important interplay of chemotaxis, extrahyphal localization, and early symbiotic interactions between bacteria and fungi (119). *L. bicolor* uses chemotaxis to attract the mycorrhizal helper bacterium *Pseudomonas fluorescens* (119), selecting for particular strains by exuding trehalose, a polyol compound that is a carbohydrate source in and around the mycorrhizal association. When trehalose is used as the sole carbon source in thin agar on glass microscope slides, fluorescent *P. fluorescens* bacteria accumulate onto and around the hyphae. This kind of attraction of the bacteria specifically to hyphae by the fungus indicates the potential for close interaction and communication, possibly signifying nutritional exchanges (119). Such signaling would be of interest in the case of class 3 EHB and in those cases in which *de novo* colonization occurs in fungal hyphae by class 2 EHB.

Once in proximity, bacteria and fungi typically communicate over shorter distances (micrometers to millimeters) via larger (>300 daltons), nonvolatile, and often specialized signals. These signals can be proteinaceous or nonproteinaceous and can diffuse through aqueous environments. We anticipate that proteinaceous signaling molecules are particularly important in pre- and early symbiotic phases of EHB-fungi associations. Fungi and many bacteria acquire nutrients via extracellular digestion, such that they secrete digestive enzymes into their local environment. These enzymes include cellulases, chitinases, glucanases, proteases, and lysozymes (151, 152). Although little has been documented about the roles of enzymes in direct bacteria-fungi interactions, chitinases have been indicated as important factors for bacterial mycophagy (152) and EHB entry into fungal cells (108).

Frontiers with Respect to Protein Secretion Systems

Bacteria rely on secretion systems to release large biomolecules into the local environment and neighboring cells. These systems can range from simple transporters to large protein complexes with specialized functions. In Gram-negative bacteria these multicomponent secretion systems have been catalogued into at least six types (type I to type VI), each differing in components and molecular mechanisms. These systems often are associated with virulence but have also been implicated in other symbiotic associations. They certainly are relevant in many EHB-fungal associations, but studies in this area are in an early phase.

Type I and type II systems secrete lipases, proteases, and beta-glucanases, which have been implicated in antifungal properties of different bacterial species (153, 154). Type II systems that secrete chitinases are essential for bacterial entry into fungal hyphae in the *Paraburkholderia-Rhizopus* endosymbiosis (class 2) (108).

Type III and type IV systems directly inject macromolecules such as proteins and DNA into host cells and have been implicated mostly in interactions with plants and animals, although there is a growing body of literature that suggests that these systems can also impact interactions with fungi. Specifically, type III secretion systems are essential for the maintenance of endosymbiotic bacteria in *Paraburkholderia-Rhizopus* interactions (107). Similarly, type III systems influence how the mycorrhizal helper bacterium, *P. fluorescens* BBc6R8, shapes colonization of pine roots by *L. bicolor* (155). In turn, *Agrobacterium tumefaciens* (syn. *R. radiobacter*) utilizes type IV systems to inject transformative plasmids into host plants (156). This transformation system has been adopted for biotechnological purposes to transform a number of fungi *in vitro* (125). Thus, type IV systems may have a yet undescribed function in mediating the transfer of genetic material from bacteria to fungi. While it is tempting to assume that some evidence of bacteria-fungi HGT may have been mediated by bacterial type IV secretion systems, particularly via EHB symbioses, the mechanisms of such gene transfer events have yet to be explored.

Type V and VI systems have received limited attention to date in EHB, although they may also play a role due to their implications in pathogenesis and interactions with eukaryotes. Type V systems secrete virulence factors and adhesins through the outer membrane, which can contribute to attachment and biofilm formation (157). These systems may be important for maintaining extracellular contact with fungi during early colonization and potentially matter for fungi-mediated translocation. Newly classified type VI secretion systems are important for virulence and are conserved across pathogenic (i.e., *Vibrio*, *Pseudomonas*), beneficial (i.e., *Rhizobium*), and environmental *Proteobacteria* (158). Because of the ubiquity and potential diversity in impacts of type VI systems, their potential roles in EHB associations are compelling for further exploration. Notably, genes for type VI secretion systems are widespread in diverse class 3 EHB (Fig. 8) (43).

Importantly, secretion systems are not the only elements relevant for EHB to enter fungal hyphae. The occurrence of *Firmicutes* and *Bacteroidetes* as EHB opens the door to exploring other means by which class 3 EHB and others with horizontal transfer via an extrasymbiotic phase can gain entry into the living cells of their fungal hosts. In the broad sense, these widespread and potent symbioses appear to be an extremely common feature in the biology of plant-associated fungi. We anticipate that future work will detect additional bacterial phyla and fungal clades involved in intimate interdomain relationships that impact the ecological modes of fungi and the hosts with which they, in turn, interact. Adopting the perspective of Wolf and Wolf (65) argues for assessing fungal holobionts—with their symbiotic bacteria taken into consideration—when exploring the phenotypes, gene expression, communication, and ecological activity of fungi. Together, these are exciting frontiers that have been enabled by a foundation of pioneering studies and the confluence of front-line methods in imaging, biochemistry, phenotyping, computation, and molecular biology.

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Necrotrophic Mycoparasites and Their Genomes

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INTRODUCTION

Mycoparasitism is a lifestyle where a living fungus (host or prey) is parasitized by and acts as a nutrient source of another fungus (mycoparasite or predator). Mycoparasitic interactions are common among fungi and, similar to plant pathogens, comprise both biotrophic as well as necrotrophic relationships. Biotrophic mycoparasites obtain their nutrition from living fungal host hyphae, with which they can live in a balanced relationship for extended stages of their life cycles, and usually show a narrow host range (1). Because of their nature, however, only few species are studied in detail and applied as biocontrol agents. Examples are *Ampelomyces quisqualis* (Ascomycota, Pezizomycotina), a natural enemy of powdery mildews, whose genome sequence has recently been released, as well as haustorial biotrophs from the Zygomycota (2, 3). In contrast to biotrophs, necrotrophic mycoparasites are more destructive and often rather unspecialized (4). They usually have wide host ranges, often comprising fungal plant pathogens, and kill their fungal prey by invasion and secretion of damaging molecules followed by feeding on the released nutrients (5, 6). These traits render these fungi useful in agriculture for the biological control of plant diseases.

The diversity of mycoparasitic fungi and the variety of mycoparasitic relationships have been reviewed elsewhere (5, 7). We will here focus on necrotrophic mycoparasites. Certain species of particular genera of Hypocreales, especially *Trichoderma* but also *Clonostachys*, are among the best studied mycoparasitic fungi. Considering that the elucidation of the mechanisms involved in mycoparasitism is among the key factors for the development and purposeful application of biocontrol agents, it is not surprising that *Trichoderma* and *Clonostachys* are contained in many biocontrol formulations (4, 8).

While the mycoparasitic nature of *Trichoderma* has been known for over 80 years (9), and several *Trichoderma* species have since been well studied at the cellular, molecular, and genomic level (10), detailed studies on *Clonostachys* are on the rise. *Clonostachys rosea* was formerly classified as *Gliocladium roseum* (11), and the latter was described as a destructive mycoparasite in the 1960s (12). In recent years, *C. rosea* emerged as an effective biocontrol agent of several important plant diseases, and the interest in this mycoparasite recently resulted in the sequencing and analysis of its genome (13). Mycoparasitic *Trichoderma* spp. and *C. rosea* are not only antagonists of other fungi, but are also capable of colonizing and establishing on living plant roots, an ability termed rhizosphere competence (14–16). Root colonization often results in beneficial effects on the plants such as growth stimulation and elicitation of plant defense. Plant growth stimulation by *Trichoderma* spp. and *C. rosea* is a well-known phenomenon (17, 18). Induction of plant defense responses by *Trichoderma* spp. is also well documented, whereas only some cases have been reported for *C. rosea* (19–21). However, based on these, it seems likely that plant defense elicitation is an important biocontrol mechanism of *C. rosea* as well.

When directly applied to specific niches like flowers, wounds, and seeds, both *Trichoderma* spp. and *C. rosea* can exert biocontrol effects by being priority colonizers of such resources and thereby preventing colonization and plant infection by pathogens. Exploitation competition or interference competition via antibiosis might

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be important mechanisms that may precede a mycoparasitic interaction. Mycoparasitism in many such cases would come too late for efficient biocontrol of pathogens infecting via the same niches, because the pathogens in such cases might infect the plant and cause disease within hours, long before mycoparasitism is established (discussed in reference 22).

Concerning mycoparasitic activities, various *Trichoderma* species such as *T. virens*, *T. atroviride*, *T. asperellum*, species from *T. harzianum* complex, as well as *C. rosea* are highly efficient in overgrowing and killing their fungal prey. To this end, they apply a combination of mechanisms that may act simultaneously or sequentially and that may vary in a species-specific and prey-dependent way. *Trichoderma* species are invasive mycoparasites that, upon contact, attach to their prey, often followed by coiling around the prey hyphae and formation of appressoria-like infection structures aiding in penetration (23–25). *C. rosea* has similar mycoparasitic traits because it can coil around hyphae and spores and infect resting structures like sclerotia of other fungi. Hyphal swellings at the points of interaction have been observed with *Fusarium oxysporum* hyphae (13), but the formation of appressoria as a general trait in *C. rosea* mycoparasitism has, to our knowledge, not been verified (13, 26, 27). To be able to feed on the prey, the mycoparasite has to degrade the prey's cell wall (Fig. 1). Hence, hydrolytic enzymes and their respective genes, whose abundance in the genomes of *Trichoderma* mycoparasites is indeed increased relative to other fungi, are traditionally regarded as key features in mycoparasitism. Attack of the fungal prey can further be supported by secreted volatile and water-soluble secondary metabolites with inhibitory or toxic action (10).

While studies on the molecular aspects of mycoparasitism often relied on the analysis of single genes, the recent sequencing of the genomes of selected mycoparasitic *Trichoderma* spp. and *C. rosea*, as well as of additional mycoparasitic species in the order Hypocreales, i.e., *Tolypocladium ophioglossoides* and *Escovopsis weberi*, paved the way for high-throughput studies. Genome analyses revealed several gene families with a mycoparasitism-related expansion, but distinct differences also emerged between the different mycoparasites (13, 28–30). Furthermore, global transcriptome studies led to the identification of gene sets being specifically activated during the mycoparasitic interaction with living fungal preys or, in the case of *T. ophioglossoides*, isolated host tissue (31–34).

In this article, we will focus on mycoparasitic species in the order Hypocreales, mainly the well-characterized *Trichoderma* and *Clonostachys* mycoparasites, for illustration of biological principles and the molecular basis of necrotrophic mycoparasitism. We will describe the knowledge gained over the years on *Trichoderma* as a "model" mycoparasite and will compare its mycoparasitic strategies with the behavior and special features of *C. rosea* and, where applicable, with other hypocrealean mycoparasites.

HYPOCREALEAN NECROTROPHIC MYCOPARASITES: SYNOPSIS OF THEIR BIOLOGY AND GENOMES

The order Hypocreales (class Sordariomycetes) comprises major lineages of mycoparasitic fungi including the well-studied *Trichoderma* and *Clonostachys* biocontrol agents as well as the more specialized mycoparasites within the genera *Escovopsis* and *Tolypocladium*. Sung et al. (35) demonstrated that shifts to mycoparasitism occurred several times during the evolution of hypocrealean fungi. For example, mycoparasitic *Clonostachys* spp. (Bionectriaceae), which are closely related to plant pathogenic *Fusarium* (Nectriaceae), likely obtained this ability after diverging from a plant-feeding host, while ancestors of *Trichoderma* and *Escovopsis* likely evolved from animal pathogens (36). As another example, next phylogenetic neighbors of highly specialized *Tolypocladium* (Ophiocordycipitaceae), a parasite of ectomycorrhizal truffles, are all pathogens of insects (30).

In the genus *Trichoderma*, most species not only are capable of mycoparasitism, but also efficiently feed on dead fungal biomass, often depending on the type of *Trichoderma* strain itself, the type of interacting fungus, and the stage of the interaction. *Trichoderma* mycoparasites have wide host ranges including plant pathogens such as the basidiomycete *Rhizoctonia solani* (Cantharellales), ascomycetes like *Botrytis cinerea, Sclerotinia sclerotiorum* (Helotiales), *Alternaria alternata* (Pleosporales), and *Fusarium* spp. (Hypocreales), but also oomycetes like *Pythium ultimum* (10). The mycotrophic behavior (the use of fungi for food) is an ancestral lifestyle of *Trichoderma* (28). However, different species developed different ecological strategies (32, 37).

The recent sequencing of genomes of hypocrealean mycotrophic fungi other than *Trichoderma*, i.e., *C. rosea* (13), *E. weberi* (29), and *T. ophioglossoides* (30), has opened up new possibilities for the investigation of the molecular mechanisms of mycoparasitism. *C. rosea*, a ubiquitous soilborne ascomycete, is known for its mycoparasitic and nematophagous abilities (38, 39).

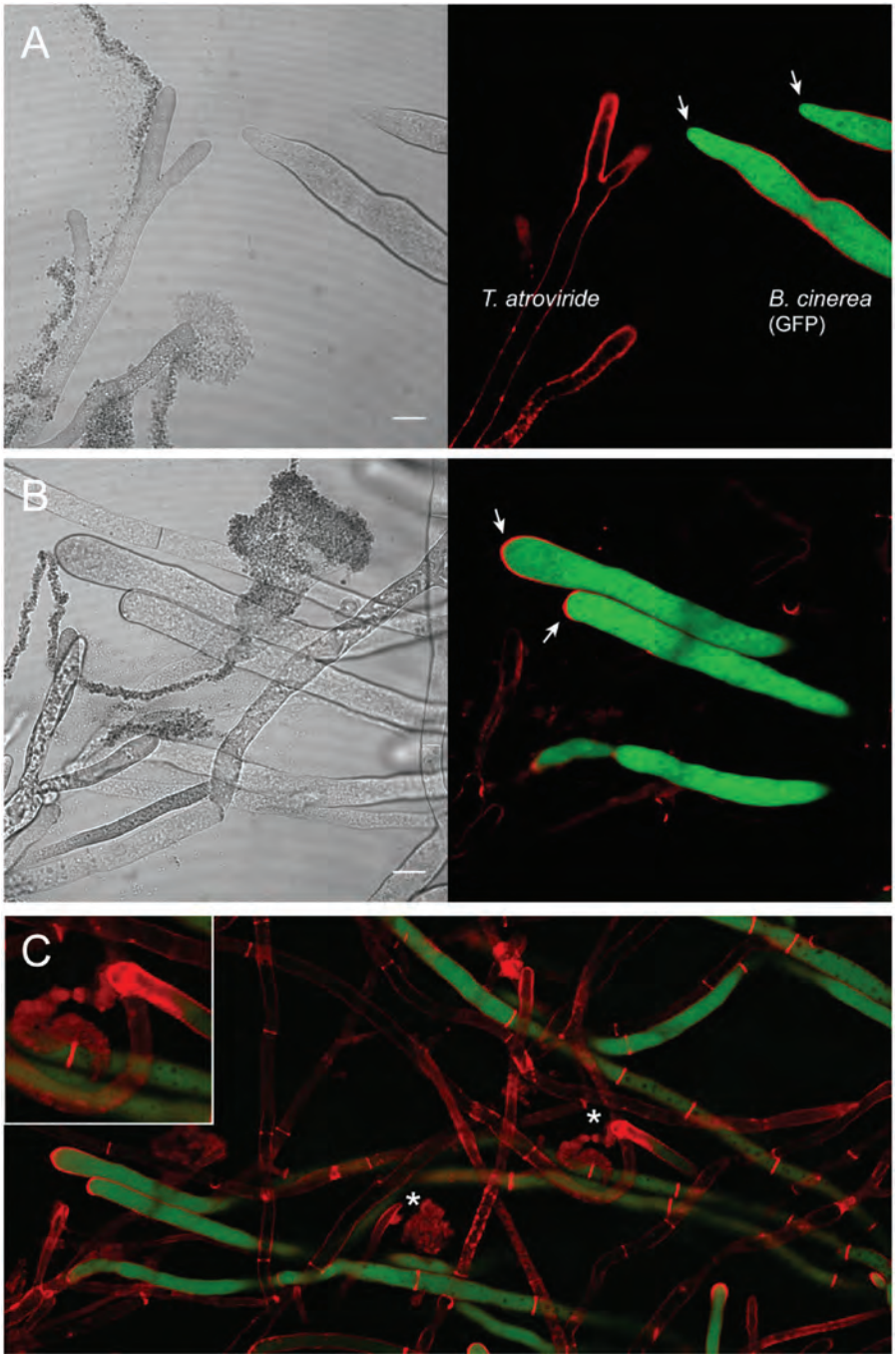


Figure 1 Interaction between hyphae of the mycoparasite *T. atroviride* and the fungal plant pathogen *B. cinerea*. (**A**) Before attack of *B. cinerea* (expressing cytoplasmic GFP) (188) by *T. atroviride*, the apical cell wall extends quickly and is only weakly stained with Congo red, a chitin-specific red fluorescent dye (arrows). (**B**) Mycoparasitic attack induces tip growth arrest in the prey hyphae, leading to tip swelling and increased chitin deposition in the apical cell wall (arrows). (**C**) Tip lysis of the prey hyphae results in cytoplasmic leakage (asterisks and inset) and use as nutrient substrate by the mycoparasite. GFP, green fluorescent protein. Scale bars: 20 μm (reprinted from *FEMS Microbiology Reviews* [189]).

Strain IK726, used for whole genome sequencing, has been isolated from barley roots infected with *Fusarium culmorum* and has proven to be highly efficient as a biocontrol agent (BCA) against a wide range of fungal and oomycete pathogens of agricultural and horticultural crops (13, 40).

In contrast to *Trichoderma* species and *C. rosea*, *Escovopsis* spp. are not generally aggressive fungi but highly selective mycoparasites with different species having different host ranges. *E. weberi* was isolated from the nests of fungi-growing leaf-cutting ants of the tribe Attini (Hymenoptera, Insecta) as a natural pathogen of *Leucoagaricus*, with which the ants share an obligate mutualism. Parasitism of *E. weberi* is specialized on the attack of leaf-cutting ant cultivated *Leucoagaricus* (Agaricales, Basidiomycota) fungi, in whose absence it grows poorly. *E. weberi* is hardly able to attack plant pathogenic fungi like *F. oxysporum*, *R. solani*, or *Sclerotium rolfsii* by which it is even agonized (29, 36). The mycoparasitic attack of *E. weberi* on *Leucoagaricus* spp. involves hook-like infection structures and penetration of the host hyphae and is further accompanied by secretion of enzymes and toxins (41, 42).

The genus *Tolypocladium* (Ophiocordycipitaceae, Hypocreales, Sordariomycetes) comprises insect pathogens as well as parasites of truffle fruiting bodies. The latter have a narrow host range restricted to the genus *Elaphomyces* (Eurotiales, Ascomycota), ectomycorrhizal fungi closely related to *Aspergillus* and *Penicillium*. The recently sequenced *T. ophioglossoides* is a commonly collected truffle parasite with a broad geographic distribution throughout many parts of the Northern Hemisphere (30).

The availability of sequenced and annotated genomes of mycoparasitic species, representing different families within the order Hypocreales, opens up new possibilities to identify key similarities and differences between their respective life strategies, which have direct bearing on the implementation of biological control in agriculture and horticulture. Genome size, structure, and gene content are heavily influenced by natural selection governed by the lifestyle and ecological niche of a species. Comparative analyses of gene content, or paralogous gene number gains or losses, are used extensively to identify genes (representing mechanisms) that are key determinants for ecological niche adaptation. The theoretical basis for this is that the long-term outcome of gene duplication and gene loss is highly constrained by the functions of the encoded proteins. Wapinski and coauthors (43) showed that genes encoding proteins involved in stress responsiveness, such as transporters, receptors, cell wall proteins, and carbohydrate-active enzymes, readily exhibit high interspecific copy number variation because this enables adaptation to diverse ecological niches. In contrast, genes encoding proteins involved in growth and cellular homeostasis show selection against copy number variation. One possible explanation is that many of these proteins are essential components of protein complexes and cellular machineries that require stoichiometric balance, i.e., the gene balance hypothesis (44).

So far, sequenced *Trichoderma* genome sizes vary between 31.7 Mbp and 39.0 Mbp and are similar to the genome size of the truffle parasite *T. ophioglossoides* (31.2 Mbp) (28, 30, 45, 46). It is notable that the species *T. longibrachiatum*, *T. reesei*, and *T. parareesei* (47) from the Longibrachiatum section have smaller genome sizes than species from Virens clade and section *Trichoderma* (Table 1). This reduction in genome size is accompanied by a lower number of protein-encoding genes. A similar but even more pronounced reduction in genome size and gene numbers occurred in *E. weberi*, whose genome with an estimated size of ~29 Mbp is among the smallest sequenced genomes of all Pezizomycotina (29). Primary metabolism-associated genes have been retained in the *E. weberi* genome, while it is depleted in carbohydrate-active enzyme-encoding genes, mainly those for plant degradation, a gene loss that is attributed to its specialization as a parasite of ant-cultivated *Leucoagaricus* spp. (29). For *C. rosea*,

Table 1 Genome sizes and gene numbers of mycoparasites in the Hypocreales order

Species	Family within Hypocreales	Genome size (Mbp)	Predicted gene number	Reference
Clonostachys rosea	Bionectriaceae	58.3	14268	13
Escovopsis weberi	Hypocreaceae	29.5	6870	29
Tolypocladium ophioglossoides	Ophiocordycipitaceae	31.2	10134	30
Trichoderma atroviride	Hypocreaceae, section Trichoderma	36.1	11863	28
Trichoderma hamatum	Hypocreaceae, section Trichoderma	38.2	12391	45
Trichoderma longibrachiatum	Hypocreaceae, section Longibrachiatum	31.7	9409	46
Trichoderma reesei	Hypocreaceae, section Longibrachiatum	34.1	9129	190
Trichoderma virens	Hypocreaceae, clade Virens	39.0	12427	28

the sequenced and annotated genome of strain IK726 (13) and the draft genome of strain 67-1 (48) were recently published. The genome size of *C. rosea* (55.4 to 58.3 Mbp) is comparable with that of some closely related *Fusarium* species, such as *F. solani* (51.1 Mbp [49]) and *F. oxysporum* (61.4 Mbp [50]).

While the draft genome of *T. ophioglossoides* has primarily been analyzed in regard to secondary metabolite biosynthetic genes and clusters, whose numbers are similar to those found in *Trichoderma* mycoparasites (28, 30), closer insights into the gene content of mycoparasitic *Trichoderma* species, *C. rosea*, and *E. weberi* are available from comprehensive comparative genome analyses. The *E. weberi* genome sequence shows a high degree of micromesosynteny with the genomes of *T. atroviride* and *T. virens*, pointing to a similar gene content but shuffled order and orientation. Of the 1,066 genes being unique to *E. weberi* relative to *Trichoderma* spp., most are of unknown function, and only 128 encode proteins with homology to proteins in other Pezizomycotina (29). Gene families exhibiting the largest expansions in *Trichoderma* spp. compared with other ascomycete fungi include Zn(2)Cys(6) transcription factors, major facilitator superfamily (MFS) transporters, short-chain alcohol dehydrogenases, S8 peptidases, and ankyrin-repeat proteins (28, 46). In *C. rosea*, the largest gene expansions compared with other fungi are among pectin/pectate lyases, glucose-methanol-choline oxidoreductases, lytic polysaccharide monooxygenases, ATP-binding cassette (ABC) transporters, polyketide synthases (PKS), and cytochrome P450 monooxygenases (CYP) (13). In the following sections, we will discuss specific gene families with relevance for the mycoparasitic lifestyle.

SENSING THE FUNGAL PREY AND ACTIVATION OF MYCOPARASITISM-RELATED SIGNALING PATHWAYS

The mycoparasitic fungus-fungus interaction is a complex, multistage process best studied in *Trichoderma*. *Trichoderma* is first being attracted by the prey, probably by chemotropism (6). Recognition of the fungal prey then leads to transcriptional reprogramming and expression of the "molecular weapons" involved in prey attack and lysis. Diffusible prey-released substances seem to induce the expression of a set of genes, including certain cell wall-degrading enzymes (CWDEs), before direct contact between the two fungi. Specific cell surface signaling mediated by prey-associated lectins mainly is responsible for attachment of *Trichoderma* to and its coiling around the prey hyphae (51–53). More recent genome-wide transcriptome studies confirmed the precontact induction of a distinct set of genes in *T. atroviride* and *T. virens* upon cocultivation with *R. solani* (31, 32). However, *T. virens* (strain Gv29-8, a gliotoxin-producing Q strain) mainly increased the expression of genes for gliotoxin biosynthesis, while *T. atroviride* strongly upregulated hydrolytic enzyme-encoding and certain secondary metabolism-associated genes (32). Studies with *T. atroviride* revealed that the contact-independent expression of CWDE-encoding genes such as *ech42* (endochitinase 42-encoding) and *prb1* (alkaline proteinase-encoding) is dependent on enzyme diffusion from the mycoparasite to its host (52–54). As a consequence of the actions of CWDEs on the fungal prey, oligosaccharides and oligopeptides are released by the prey that are then recognized by *Trichoderma* receptors and thereby act as inducers. The model is supported by the expression of several G-protein-coupled receptor (GPCR) genes in response to the host. These include members of the PAQR (progestin-adipoQ receptor), the Stm1-like (putative nitrogen sensors), and the PTH11-like (related to PTH11, which is required for appressorium formation and pathogenicity in *Magnaporthe oryzae*) families whereby differences in the transcriptional regulation between *T. atroviride* and *T. virens* are clearly evident in the latter two GPCR families (55). Two predicted PTH11-encoding genes are also induced in *T. ophioglossoides* during growth on truffle cell wall-containing media (34), emphasizing the importance of PTH11-type receptors in hypocrealean mycoparasites.

Heterotrimeric G-Protein Signaling

The heterotrimeric G-protein signaling pathway is conserved in eukaryotes and usually involves a GPCR, a heterotrimeric G protein (composed of the Gα, Gβ, and Gγ subunits), and an effector. In the classic paradigm, ligand binding to the GPCR causes the exchange of the GDP bound to the Gα subunit with GTP, which results in release of the Gβγ dimer. The released dimer and/or activated Gα then interact with downstream effectors, which in fungi often are mitogen-activated protein kinase (MAPK) cascades or the cAMP-generating adenylate cyclase (56). Of the three Gα proteins encoded in *Trichoderma* spp., two play essential roles in mycoparasitism, i.e., the adenylate cyclase-inhibiting (Tga1 in *T. atroviride*, TgaA in *T. virens*) as well as the adenylate cyclase-activating (Tga3 in *T. atroviride*) subunits (57–60). However, although *T. atroviride* Tga1 and *T. virens* TgaA are highly conserved at the amino acid level, quite different phenotypes emerged from deletion of the corresponding genes. While *T. virens*

TgaA does not affect the mycoparasitic activity against *R. solani* but governs the colonization of *S. rolfsii* sclerotia (58), *T. atroviride* Tga1 regulates growth and asexual sporulation and is essential for activating attack and overgrowth of different fungal hosts including *R. solani* (59). Similarly, Tga3 affects growth and conidiation in *T. atroviride* and impacts secondary metabolite production and extracellular chitinase activity. The avirulent phenotype of Δ*tga3* mutants further resulted from their inability to adhere to and coil around host hyphae, suggesting a role of the Tga3 protein in the transmission of signals that regulate prey recognition (60).

From the 65 and 76 putative GPCRs encoded in the *T. atroviride* and *T. virens* genomes, respectively (61), two *T. atroviride* members of the fungal cAMP receptor-like GPCR family have been functionally characterized so far. While silencing of the *gpr2* gene did not result in any phenotype (62), Gpr1 is essential for the antagonistic activity of *T. atroviride*. *gpr1*-silenced strains share several phenotypic alterations with Δ*tga3* mutants such as the inability to recognize and attack living fungal hosts (63). *gpr1* mutants are unable to respond to the presence of a living prey by expressing chitinase- and protease-encoding genes; consequently, prey hyphae remain completely intact and undamaged upon confrontation with the *gpr1*-silenced mutants (63). Although a physical interaction between Gpr1 and Tga3 could not be proven yet, their concerted action is further corroborated by reduced intracellular cAMP levels in both *tga3* and *gpr1* mutants and by the fact that addition of extracellular cAMP can rescue their defect in host adherence and infection structure formation (60, 63).

The cAMP Pathway

Upon stimulation by adenylate cyclase-activating Gα proteins, the membrane-bound adenylate cyclase enzyme synthesizes cAMP from ATP. cAMP then acts on a cAMP-dependent protein kinase (PKA), consisting of two regulatory and two catalytic subunits, that upon activation regulates gene expression by phosphorylating respective target proteins (64). Deletion of the single adenylate cyclase gene *tac1* in *T. virens* lowered the intracellular cAMP level to below the detection limit and resulted in mutants with strongly reduced growth on solid media and loss of biocontrol activity against plant pathogens such as *S. rolfsii, R. solani*, and *Pythium* spp. (65).

Application of exogenous cAMP stimulates coiling, whereas a reduced intracellular cAMP level as found in, e.g., *T. atroviride* Δ*tga3* mutants (60) results in a loss of infection structure formation. The synthesis of *N*-acetyl-β-D-glucosaminidase (NAGase) by *T. harzianum*, in contrast, is repressed by substances that increase the intracellular levels of cAMP (e.g., dinitrophenol, caffeine, aluminum tetrafluoride) (66). Accordingly, low cAMP levels and PKA activity elevate the transcription of *prb1* and *ech42* in *T. atroviride* (57), highlighting that multiple signals and effector pathways are involved in the regulation of mycoparasitic responses in *Trichoderma*.

MAPK Cascades

MAPK pathways, being among the most prominent signal transduction systems in fungi, contain a highly conserved core structure with a three-kinase module. Input signals lead to activation of the MAPK cascade by sequential phosphorylation from a MAP kinase kinase kinase (MAPKKK) across a MAP kinase kinase (MAPKK) to a MAP kinase (MAPK) that transmits it to downstream target proteins such as transcription factors, thereby generating a specific response (67).

Trichoderma spp. have MAPK members common in filamentous fungi that act in the pheromone response/pathogenicity (Tmk1/TmkA), the cell wall integrity (Tmk2/TmkB), and the osmosensing (Hog1/Tmk3) pathways (28). The functions of all three types of conserved MAPKs have been studied in mycoparasitic *Trichoderma* species. The *T. virens* and *T. atroviride* homologues (Tmk1/TmkA) of the yeast pheromone response MAPK are involved in mycoparasitism (68–70), a situation similar to plant pathogenic fungi where Fus3/Kss1 homologues have been described as effectors of virulence-associated processes (71). *T. atroviride* Tmk1 governs the production of CWDEs and antifungal metabolites such as certain peptaibols and 6-pentyl-α-pyrone (6-PP) as well as aerial hyphae formation and conidiation. Δ*tmk1* mutants have enhanced chitinase and antifungal activities, but nevertheless exhibit reduced mycoparasitic activity (70). Comparative analyses of Δ*tmk1* and Δ*ste12* mutants (missing the Ste12 transcription factor whose orthologue in yeast is a target of the Fus3 and Kss1 MAPKs) revealed an essential role of Ste12 in mediating the outcomes of the Tmk1 MAPK pathway, such as regulation of the mycoparasitic activity, hyphal fusion, and vegetative growth (72). In *T. virens*, the function of the Tmk1 homologue is strain specific. Respective deletion mutants in a gliotoxin-producing "Q" strain of *T. virens* have increased biocontrol activity against *R. solani* accompanied by an enhanced expression of mycoparasitism-related genes, the latter being similar to *T. atroviride* (68). In contrast, signaling through TmkA in a "P"

(gliovirin-producing) strain does not affect mycoparasitism of *R. solani* but stimulates antagonism against *S. rolfsii* (69).

Tmk2/TmkB, a homologue of the yeast cell wall integrity MAPK Slt2, is involved in cell wall integrity in both *T. reesei* and *T. virens* (73, 74). *T. virens* TmkB in addition has overlapping functions with TmkA (similar to Δ*tmkA* mutants, Δ*tmkB* mutants have a reduced ability to overgrow *S. rolfsii* while retaining their mycoparasitic activity against *R. solani*), suggesting shared downstream targets (73).

The Hog1 MAPK is a central component of the high osmolarity glycerol (HOG) pathway that mediates responses to hyperosmotic shock and other stresses in fungi (75). Accordingly, *T.* cf. *harzianum* Hog1 and the *T. atroviride* homologue Tmk3 participate in high-osmolarity resistance and the respective mutants are sensitive to osmotic and oxidative stress (76, 77). Hog1 positively affects the antagonistic activity of *T.* cf. *harzianum* against certain plant pathogens by neutralizing stress agents such as reactive oxygen species (ROS) produced by fungal preys (76).

The Global Regulators Lae1 and Vel1

The putative *S*-adenosylmethionine-dependent methyltransferase LaeA of *Aspergillus nidulans* forms a trimeric protein complex with VeA and VelB that serves as a global regulator of secondary metabolism and morphogenesis (78). In *T. virens*, Vel1 (VeA homologue) regulates not only conidia and chlamydospore formation, but also secondary metabolism and mycoparasitism (79). Similar functions have been reported for *T. atroviride* Lae1 (LaeA homologue) where *lae1* deletion mutants are completely avirulent, which is accompanied by a significant underexpression of several genes normally being induced during the mycoparasitic interaction. Δ*lae1* mutants are further unable to defend themselves against other fungi indicated by the finding that their growth is suppressed by the tested plant pathogens and that the mutants are more sensitive to oxidative stress (80). The overlapping functions of Lae1 and Vel1, together with the fact that Vel1 is highly conserved among *Trichoderma* species, make it likely that the Lae1-Vel1-Vel2 complex acts as a master regulator of *Trichoderma* mycoparasitism.

CELL WALL-DEGRADING ENZYMES AND THEIR ROLE IN MYCOPARASITISM

The fungal cell wall is a dynamic structure that protects the cell from environmental stresses while allowing it to interact with its environment (81). The exact biosynthesis and composition vary between fungal species and have been studied in very few systems, but the major constituents include covalently cross-linked chitin, glucans, mannans, and glycoproteins (81, 82). Production of hydrolytic enzymes that permeabilize and degrade the fungal cell wall is one of the key steps in the successful attack of the fungal prey by necrotrophic mycoparasites. Druzhinina et al. (83) showed that enzymes degrading poly- and oligosaccharides (carbohydrate-active enzymes or CAZymes), small secreted cysteine-rich proteins (SSCPs), and proteins with unknown function comprise more than 60% of the predicted secretomes of *T. reesei*, *T. atroviride*, and *T. virens*. In contrast, enzymes with hydrolytic activity on other polymers (proteases, lipases, nucleases, and phosphatases) and orphan proteins (proteins without orthologues outside the *Trichoderma* genus) are present in lower numbers.

Chitinases

Chitin is a β-1,4-linked *N*-acetylglucosamine homopolymer that provides structural rigidity to the fungal cell wall (82). Although chitin fibrils are mostly located in the inner layers of the cell wall close to the plasma membrane, chitin appears to be an important target for degrading enzymes during the mycoparasitic attack. Fungal chitinases are classified as glycoside hydrolase family 18 (GH18), which is divided into clusters A, B, and C and further subdivided into subgroups A-II to A-V, B-I to B-VI, and C-I to C-II based on the amino acid sequence of the GH18 module (84–87).

The importance of chitinases for *Trichoderma* spp. is reflected by the high number of GH18 genes present in their genomes (28, 45, 46). Comparative analyses with other filamentous ascomycetes revealed that the increased number of chitinase genes in *Trichoderma* spp. and the truffle-parasite *T. ophioglossoides* (Table 2) is specifically related to subgroups B-I/B-II and C-I/C-II (34, 88, 89), indicating that the corresponding enzymes play important roles in these mycoparasites. Interestingly, *C. rosea* differs substantially from mycoparasitic *Trichoderma* spp. because the genome contains only a few genes in these subgroups (two genes each for B-I/B-II and C-I/C-II) (13, 90). Finally, the *E. weberi* genome has a similar number of chitinases as *T. reesei* with many subgroup B-I/B-II but few subgroup C-I/C-II genes (29), indicating fundamental differences in the role of fungal cell wall degradation between the different mycoparasites.

Characterized *Trichoderma* cluster B chitinases show a preference for endoactivity (91–93) and possibly pro-

Table 2 Predicted number of glycoside hydrolase family 18 genes in mycoparasitic and nonmycoparasitic ascomycete fungi

Species	Cluster A	Cluster B	CHIT36[a]	Cluster C	ENGases	Total
Mycoparasitic species						
Clonostachys rosea	8	2	1	2	1	14
Escovopsis weberi	5	6	1	2	2	16
Tolypocladium ophioglossoides	7	6	–	10	1	24
Trichoderma longibrachiatum	7	6	1	3	2	19
Trichoderma reesei	7	6	1	4	2	20
Trichoderma virens	8	10	1	15	2	36
Trichoderma hamatum	6	10	1	5	2	24
Trichoderma atroviride	7	10	1	9	2	29
Nonmycoparasitic species						
Botrytis cinerea (plant pathogen)	3	4	–	1	2	10
Coccidioides immitis (animal pathogen)	4	2	–	2	1	9
Magnaporthe oryzae (plant pathogen)	9	2	–	2	2	15
Neurospora crassa (saprobe)	5	2	–	3	2	12

[a]CHIT36 represents a cluster B chitinase of bacterial origin transferred to fungi through horizontal gene transfer (84).

vide an initial attack on the cell wall chitin of the fungal prey (94). Many of these cluster B chitinase genes are induced during interactions with other fungi or by fungal cell wall material in *Trichoderma* spp. (86, 93, 95–97) as well as *C. rosea* (90, 98, 99).

Due to sequence similarity with the secreted killer toxin from certain yeast species (100), some cluster C chitinases are hypothesized to act in fungal-fungal interactions through a killer toxin-like mechanism to enhance the permeability of antifungal compounds into antagonist cells (86). Recent studies support this idea, because deletion of the killer toxin-like chitinase genes *chiC2-2* in *A. nidulans* and *chiC2* in the mycoparasite *C. rosea* resulted in lower fungal growth inhibitory activity of culture filtrates (90, 101). The growth inhibitory effect of the *chiC2* deletion in *C. rosea* was observed for both *B. cinerea* and *R. solani*, but not for *Fusarium graminearum*, which suggests some specificity in the action of CHIC2. Induced gene expression of cluster C chitinase genes in *Trichoderma* spp. and *T. ophioglossoides* during exposure to fungal cell wall material and during mycoparasitic interactions indicates a similar role, although some members are also regulated developmentally and by nutritional stimuli (34, 86, 102, 103).

The best studied chitinases in mycoparasitic fungi are Ech42 orthologues (Ech42 in *T. atroviride*, Chit42 in *T.* cf. *harzianum*, Chi18-5 in *T. reesei*, Cht42/Tv-Ech1 in *T. virens*, ECH42/CHI1 in *C. rosea*) that belong to cluster A. *T. atroviride* Ech42 was initially regarded as a key enzyme for mycoparasitism based on induced gene expression during fungal interactions and gene deletion studies (104–106). However, it is also induced during starvation and autolysis, suggesting a role in self- and non-self-cell wall degradation rather than mycoparasitism *per se* (107). A similar situation is seen for the two GH20 NAGase proteins NAG1 and NAG2 of *Trichoderma* spp. that were initially considered as specifically important for mycoparasitism. Now, expression and gene deletion data suggest that they cleave *N*-acetylglucoseamine dimers into monomers for nutritional purposes, irrespective of the chitin source (94, 107, 108). *Trichoderma* spp. contain two paralogous enzymes, Eng18A and Eng18B, in the GH18 subgroup B-V. They show endo-β-*N*-acetylglucosaminidase (ENGase) activity and are involved in deglycosylation of glycoproteins (109, 110). Eng18A is secreted and is hypothesized to be involved in *N*-deglycosylation of extracellular cellulases in *T. reesei* (110), while Eng18B functions in the endoplasmic reticulum-associated degradation of misfolded glycoproteins as shown for *T. atroviride* (109). Deletion of the *Eng18B* gene results in a severe secretion defect that abolishes the antagonistic ability of *T. atroviride* toward *B. cinerea* in confrontation assays (111).

Chitosanases

Chitosan is a totally or partially deacetylated derivative of chitin (112) and chitosanases are chitosan *N*-acetylglucosaminohydrolase enzymes that catalyze endohydrolysis of beta-linkages between D-glucosamine residues. While most fungi have only one or two chitosanase (GH family 75)-encoding genes, the mycoparasitic *Trichoderma* spp. and *C. rosea* have five (13, 28). Several studies showed an enhanced production of chitosanases by *Trichoderma* species (113, 114), but details regarding their transcriptional regulation or their enzymatic properties are lacking.

Glucanases

The major fibrillar polymer in fungal cell walls is β-(1,3)-glucan, or laminarin, which is degraded by β-1,3-glucanases. While chitin is arranged in regularly ordered layers, β-(1,3)-glucan fibrils, which represent more than 60% of the fungal cell wall, are ordered in an amorphic manner (82). In *T.* cf. *harzianum*, two distinct β-1,3-glucanases (BGN13.1 and LAM1.3) are induced during growth on media containing fungal cell wall polymers or from dual cultures with host fungi (115, 116). In *C. rosea*, β-1,3-glucanase activity and gene expression are induced during growth on media containing laminarin, *Pythium* sp. cell wall material, and homogenized cucumber roots (117). Similarly, two putatively secreted β-glucanase-encoding genes are upregulated in *T. ophioglossoides* cultured on media containing truffle tissue (34).

β-1,3-Glucanases have so far been attributed to the GH families 16, 17, 55, 64, and 81. Numbers of GH55- and GH64-encoding genes are increased in mycoparasitic *Trichoderma* spp. in comparison with other filamentous fungi (28), and four genes coding for GH16 members are strongly upregulated in *T. atroviride* during the mycoparasitic attack of *R. solani* (32).

Trichoderma spp. also produce β-1,6-glucanases that hydrolyze β-1,6-glucans, which constitute a minor portion of the fungal cell wall polymers. β-1,6-Glucanases belong to GH families 5 and 30, and are suggested to play an important role in mycoparasitism against a wide variety of oomycetes (whose cell walls are mainly composed of β-1,3- and β-1,6-glucans and cellulose) and fungi (115, 118, 119). In *T.* cf. *harzianum* CECT 2413, β-1,6-glucanase activity was detected during growth on chitin, pustulan, nigeran, and fungal cell walls, and three extracellular β-1,6-glucanases (BGN16.1, BGN16.2, and BGN16.3) have been purified from this strain (115, 118). BGN16.3 is expressed during the interaction of *T.* cf. *harzianum* CECT 2413 with *B. cinerea* (120). *Tvbgn3*, a β-1,6-glucanase-encoding gene from *T. virens*, is induced in the presence of the oomycete *P. ultimum*, indicating that this enzyme might be involved in mycoparasitism. Deletion and overexpression of *Tvbgn3* resulted in reduced or enhanced ability to inhibit the growth of the plant pathogen, respectively (119).

Cellulolytic Enzymes

As mentioned previously, a major fraction of oomycete cell walls consists of cellulose and hemicelluloses. Therefore, economically important plant pathogenic oomycetes, such as *Pythium* spp., may be combated using microbial agents that are able to produce cellulolytic enzymes. Cellulose is a polysaccharide consisting of a linear chain of several hundred to many thousands β-1,4-linked D-glucose units. Inglis and Kawchuk (121) tested 14 mycoparasitic and biocontrol fungi for their ability to degrade and utilize the cell walls of the oomycete *P. ultimum*. The authors showed that degradation of *P. ultimum* cell walls was successful by several *Trichoderma* species, but not by *C. rosea*. Upon direct interaction, *T. reesei* is able to produce holes characteristic of cell wall-degrading enzyme activity in *P. ultimum* cell walls (122). Thrane et al. (123) reported on *T.* cf. *harzianum* T3 having a significant ability to control *Pythium* damping-off of cucumber seedlings and that endo-β-1,3-glucanase and cellulase activities are induced by the presence of *Pythium*. Purified endo-β-1,3-glucanase and cellulase from *Trichoderma* were inhibitory to the germination and growth of encysted *Pythium* zoospores. However, a study using the *T. reesei* cellulase-negative mutant QM9978, which still is able to overgrow *P. ultimum* in plate confrontation assays as well as to protect plants, showed that cellulase gene expression seems not to be required for the observed antagonistic activity (124). In contrast to the diversity of carbohydrate-active enzymes produced by the broad-host-range mycoparasite *Trichoderma* spp., the specialized mycoparasite *E. weberi* lacks all cellobiohydrolases, xylanases, and auxiliary proteins like polysaccharide monooxygenases and swollenins while retaining chitinolytic enzymes. This suggests that *E. weberi* has lost the ability to feed on lignocellulosic plant material, possibly as a consequence of the specialization and coevolution of *E. weberi* with its host fungus (29).

Proteases

Many genes that encode proteases are expressed before and during contact with a prey in different *Trichoderma* spp. and some proteases, including aspartyl proteases, serine proteases, and subtilisin-like proteases, have been shown to be involved in mycoparasitic action (10, 125). Troian et al. (126) found that the aminopeptidase encoding gene *s9* is downregulated during the interaction of *T.* cf. *harzianum* 303/02 with *S. sclerotiorum*, whereas the trypsin-like protease-encoding gene *pra1* is upregulated during the interaction with host mycelia, sclerotia, and apothecia, and the serine protease gene *sprt* is slightly upregulated mainly in the presence of apothecia and mycelia. Hence, the trypsin-like and serine proteases may participate in the degradation of structural cell wall proteins to destabilize host cell integrity and facilitate penetration and colonization of *T.* cf. *harzianum* (126).

Trichoderma spp. have one of the largest sets of proteases among fungi (83). A total number of 383 (4.2% of all predicted protein-coding genes), 445 (3.75%), and 479 (3.85%) protease genes are predicted in the *T. reesei*, *T. atroviride*, and *T. virens* genomes, respectively, from which around 20% possess a signal peptide and are therefore entering the secretory pathway (83). Families such as the subtilisin-like proteases of the S8 family as well as dipeptidyl (S9) and tripeptidyl peptidases (S33) are expanded in *T. virens* and *T. atroviride* mycoparasites compared with *T. reesei* (28). Several extracellular proteases (mostly aspartyl and subtilisin-like proteases, dipeptidyl and tripeptidyl peptidases, and metalloproteases), but also transporters for oligopeptides and amino acids, are expressed by all three *Trichoderma* species during the attack of *R. solani* (31, 32). In *C. rosea*, 15 different protease genes are induced during growth on *S. sclerotiorum* sclerotia (33). The diverse substrate specificities of proteases produced by *Trichoderma* and *C. rosea* may act synergistically for efficient protein degradation.

THE ROLE OF SECONDARY METABOLITES IN THE MYCOPARASITIC INTERACTION

Secondary metabolites are low-molecular-weight compounds that are, unlike metabolites from primary metabolism, not directly essential for growth. Fungal secondary metabolites serve survival functions by supporting the fungus in competition against other microbes, in self-protection and defense, and have important roles in communication and interaction with other organisms (127, 128).

The mycoparasitism of *Trichoderma* and *Clonostachys* species is at least partly effective because of a strong ability of these fungi to produce and release a plethora of secondary metabolites of which several not only serve in chemical warfare against their competitors and fungal preys, but also exhibit plant-growth-promoting and plant-resistance-inducing activities (129). Secondary metabolites seem to play a crucial role in *E. weberi* as well. This specialized mycoparasite kills its ant-cultivated host fungi from a distance, thereby likely involving secreted toxins (29).

Since the discovery of gliotoxin in the 1930s (130, 131), numerous secondary metabolites have been reported from *Trichoderma* species. These comprise a diverse array of substances like nonribosomal peptides (such as peptaibiotics, epipolythiodioxopiperazines, and siderophores), polyketides, terpenoids, steroids, and pyrones; however, progress in elucidating the genes involved in their biosynthesis has been made only during recent years.

Genome analyses revealed that the sequenced mycoparasitic *Trichoderma* species and *C. rosea* are especially enriched in secondary metabolism-related genes, compared with most other filamentous ascomycetes (13, 28). Of these, nonribosomal peptide synthetases (NRPSs) are enriched in the sequenced *T. virens* strain Gv 29-8 genome, while PKSs are enriched in *C. rosea* strain CBS125111 (13), which may suggest fundamental differences between *Trichoderma* spp. and *C. rosea* in their secondary metabolite production spectra.

Secondary metabolism-related genes in *Trichoderma* spp., *C. rosea*, *T. ophioglossoides*, and *E. weberi* are mostly arrayed in multigene biosynthetic gene clusters reminiscent of bacterial operons, a situation typical for filamentous fungi (132). Usually all the genes necessary to synthesize, regulate, and self-protect the compound-producing fungus from the bioactive properties of a specific secondary metabolite are contained in a respective gene cluster (132). Interestingly, half of the secondary metabolism-related genes present in *T. atroviride* and *T. virens* are unique for the respective species and occur within nonsyntenic islands of the genome, indicating their origin by recent genome rearrangements (28).

Nonribosomal Peptides

Nonribosomal peptides (NRPs) are peptide secondary metabolites being synthesized independent of the ribosome by multimodular NRPS enzymes. NRPs constitute a diverse group comprising linear, cyclic, and/or branched structures of various lengths that usually contain both proteinogenic and nonproteinogenic amino acids (133). The major NRP groups produced by *Trichoderma* and *Clonostachys* species are peptaibiotics, epidithiodioxopiperazines, and siderophores.

Peptaibiotics are linear or, in a few cases, cyclic NRPs characterized by the presence of the nonproteinogenic amino acid alpha-aminoisobutyric acid (Aib). The majority of all known peptaibiotics have been described for *Trichoderma* spp. (134, 135) with suzukacillin and alamethicin having been the first substances described (136, 137). The antibiotic activity of peptaibiotics is based on their helical structures allowing them to insert into membranes and form ion channels and pores in a concentration-dependent manner resulting in perturbation of the membrane bilayer, as was recently shown for alamethicin (138, 139). Peptaibiotics contribute to the antagonistic action of *Trichoderma* mycoparasites, and synergism between CWDEs and these NRPs results in a combined enzymatic degradation of the host cell wall and membrane permeabilization resulting in efficient cell lysis and leakage (140, 141). In addition, peptaibiotics like *T. pseudokoningii*-derived trichokonins

induce apoptotic cell death in fungal hosts (142), and *T. virens* 18- and 20-mer peptaibols are able to induce systemic resistance responses in plants (143, 144). Similarly, a mixture of peptaibiotics was found to be produced by *C. rosea* that contribute to the antifungal activity toward the plant pathogen *S. sclerotiorum* (38). Three NRPSs with 7, 14, and 18 to 20 modules, that are responsible for the production of peptaibiotics, are encoded in the *Trichoderma* genomes (145). The genome of *C. rosea* also harbors several putative peptaibiotics assembly lines with the largest comprising 20 modules, whose specificity signatures correspond well to the structure of the antimicrobial peptaibiotics isolated from this fungus (13). Supporting the finding that peptaibiotics biosynthesis is a characteristic trait of mycoparasitic lineages of Hypocreales, three large peptaibiotic gene clusters have been identified in the *T. ophioglossoides* genome and two peptaibiotic synthases in the draft genome of *E. weberi* (29, 30).

Epipolythiodioxopiperazines (ETPs) are cyclic-peptide-derived toxins characterized by a diketopiperazine ring whose disulfide bridge can cross-link proteins via cysteine residues and generate ROS through redox cycling (146). Gliotoxin is the best studied ETP member, originally isolated from *T. virens* (130, 131). *T. virens* "Q" strains are gliotoxin producers, while "P" strains are incapable of gliotoxin biosynthesis but instead produce gliovirin, another ETP. In biocontrol tests, "P" strains were more effective against the oomycete *P. ultimum* because gliovirin is highly toxic to this pathogen, whereas "Q" strains gave better control of *R. solani* (147). Similar to peptaibiotics, gliotoxin was shown to exhibit synergistic action with an endochitinase in inhibiting the germination of *B. cinerea* spores (148). The *T. virens* gliotoxin gene cluster consists of eight genes with GliP, a NRPS dioxopiperazine synthase, being the core enzyme (149). Disruption of the *gliP* gene in *T. virens* Gv29-8 led to a loss of gliotoxin production and to an attenuation of the mycoparasitic activity against *P. ultimum* and *S. sclerotiorum*; however, mycoparasitism of *R. solani* was independent of *gliP* (150).

ETPs produced by *Clonostachys* spp. comprise verticillin A, the verticillin-type metabolites gliocladine A, C, D, and E, which have antinematodal activity, and the antimicrobial compound glioperazine (151). Analysis of the *C. rosea* genome revealed one putative ETP synthetase (NPS1) presumably involved in the biosynthesis of glioperazine or a related metabolite (13).

Polyketides and Terpenoids

Polyketides include substances with antibiotic activity implicated in the competition and communication between microbes. The biosynthesis of polyketides involves PKSs that perform successive decarboxylative condensations of simple building blocks such as acetyl coenzyme A (CoA) and malonyl-CoA (128).

The mycoparasites *T. atroviride* and *T. virens* are rich in PKS genes because 20 putative PKS gene clusters could be identified in their genomes in addition to the PKS clusters being responsible for conidial pigment biosynthesis (152). Deletion of *pks4* in *T. reesei* revealed the corresponding gene as being responsible for the green pigmentation of conidia and the nonmelanized structures of fruiting bodies, for conidial cell wall stability, and for the antagonistic abilities of *T. reesei* against other fungi. Pks4 also affects the expression of other *pks* genes of the fungus and the production of volatile organic compounds (VOCs) (153). Certain strains of *C. rosea* produce TMC-151-type polyketide antibiotics with antibacterial properties (154), which are unique to *Clonostachys* spp. (155). The truffle mycoparasite *T. ophioglossoides* has been reported to produce the polyketide ophiocordin. In total, 38 secondary metabolism-associated clusters were identified in the *T. ophioglossoides* draft genome sequence harboring 15 PKSs and two PKS-like genes, 15 NRPSs and 6 NRPS-like genes, three hybrid NRPS-PKS genes, and four terpene synthase genes (30). The 17 putative secondary metabolite clusters present in the small genome of *E. weberi* are predicted to mainly harbor terpene synthase genes, some of which are upregulated when *E. weberi* is growing toward its host (29).

Trichoderma-derived terpenoids, several of which have antifungal activity, include volatile terpenes, the diterpene harziandione, the trichothecenes trichodermin and harzianum A, and the triterpene viridin (156, 157). Their biosynthesis involves terpene synthases/cyclases as key enzymes, with seven and 11 respective genes being encoded in the *T. atroviride* and *T. virens* genomes, respectively (152). Characterization of the *tri* gene cluster responsible for harzianum A biosynthesis in *T. arundinaceum* revealed a role of this nonphytotoxic trichothecene in the antagonistic activity against *B. cinerea* and *R. solani* and in elicitation of defense-related gene expression in tomato plants (158, 159). The *C. rosea* genome encodes eight putative terpene synthases (13), but functional characterization is lacking.

Volatile Organic Compounds

VOCs are defined by having high vapor pressure at room temperature and low water solubility. In contrast to polar water-soluble substances, which remain more closely associated with the secreting fungus, VOCs are volatile and able to travel over longer distances.

Trichoderma-derived VOCs comprise pyrones, mono- and sesquiterpenes, alcohols, ketones, furans, alkanes, and alkenes whose production is species specific and coincides with certain developmental stages such as sporulation (157). One of the first VOCs isolated from *Trichoderma* is 6-PP. 6-PP has antifungal and plant growth-promoting activities and it is produced by certain *Trichoderma* species only; it is, for example, missing in the commercially used *T.* cf. *harzianum* strains T22 and T39 (160). The biosynthesis of 6-PP has been studied in *T. atroviride* IMI206040, revealing that it is derived from linoleic acid via the oxidation product 13-hydroperoxide-diene (13-HPOD) (161). Comparative genome analysis revealed a lipoxygenase gene in *T. atroviride* but not in *T. virens* and *T. reesei*, which is in accordance with the fact that 6-PP is one of the major secondary metabolites produced by *T. atroviride* but not the other two species (28). The lipoxygenase-encoding gene shows upregulation during the interaction of *T. atroviride* with *R. solani* (32) and, similarly, 6-PP production is elicited by the presence of *R. solani* or *B. cinerea* (161, 162). Production of 6-PP is correlated with the aggressiveness of *Trichoderma* toward its fungal prey. 6-PP is able to inhibit mycotoxin production in *Fusarium* by a still unknown mechanism and to stimulate plant growth and defense responses (129, 163).

TOLERANCE OF THE MYCOPARASITE TOWARD TOXINS, HYDROLYTIC ENZYMES, AND ROS

During the mycoparasitic interaction, the fungal prey is not a passive victim but responds to the attack by secreting secondary metabolites, enzymes, and ROS, and a successful mycoparasite must be able to cope with this counterattack (10). For example, *C. rosea* is able to tolerate the mycotoxin zearalenone (ZEA) when interacting with *F. graminearum*. Plant pathogenic *Fusarium* spp. such as *F. graminearum* and *F. culmorum* causing *Fusarium* head blight and foot rot in wheat are known producers of various mycotoxins. One of those, the polyketide ZEA, has strong antifungal properties and is hypothesized to provide *Fusarium* with a competitive advantage against other fungi (164).

Following screening of 150 fungal species for their ability to metabolize ZEA, *C. rosea* was shown in 1988 to be able to open the ring structure of ZEA and thereby detoxify this mycotoxin (165). Takahashi-Ando et al. (166) cloned and characterized the *zhd101* gene from *C. rosea* which encodes a zearalenone lactonohydrolase responsible for ZEA detoxification. The resulting cleavage product was highly reduced in toxicity and was similar to the compound previously characterized by El-Sharkawy and Abul-Hajj (165). Subsequently, *zhd101* was identified in 10 different *C. rosea* strains with the encoded zearalenone lactonohydrolase enzyme differing in only one amino acid between the strains (167). The *zhd101* gene is constitutively expressed but can be induced by exposure to ZEA in a concentration-dependent manner, which indicates that ZHD101 may have additional functions other than ZEA detoxification (167).

Deletion of *zhd101* significantly reduces the ability of *C. rosea* to detoxify ZEA (164, 167). When tested in confrontation assays against ZEA-producing *F. graminearum*, Δ*zhd101* mutants had clearly reduced antagonistic activities compared with the *C. rosea* wild type. However, when confronted with a *Fusarium* mutant unable to produce ZEA due to a knockout of the *pks4* gene, the antagonistic activities of *C. rosea* Δ*zhd101* mutants and wild type were similar (167). Biocontrol assays with wheat seedlings further showed that ZEA detoxification by *C. rosea* is at least, in part, important for the successful outcome of the biocontrol interaction. However, other biocontrol mechanisms are also involved (167).

The *zhd101* gene is also present in strains of *C. rosea* f. *catenulatum* and in *T. aggressivum*, a mycoparasite and pathogen of mushroom farms (168). Comparison of *zhd101* expression and the capability of ZEA detoxification between *T. aggressivum*, *C. rosea* f. *rosea*, and *C. rosea* f. *catenulatum* revealed clear differences in *zhd101* expression profiles and in duration required for ZEA breakdown (168). Studies with *C. rosea* IK726 showed a 1,000-fold increase in *zhd101* expression already 2 hours after exposure to ZEA, which at later time points, however, rapidly declined (169). Such differences could be important for the ability of different strains to effectively control ZEA-producing fungi in biocontrol interactions.

Transcriptomic profiling based on suppression subtractive hybridization revealed specific genes being expressed in *C. rosea* upon exposure to ZEA (169). Besides *zhd101*, these comprise two ABC transporters belonging to subfamily G (*abcG5*-group I and *abcG29*-group V) (13, 170), suggesting that these are involved in the tolerance of *C. rosea* to ZEA (169). Subfamily G of ABC transporters is classified as a pleiotropic drug resistance transporter and is known to contribute to drug resistance or fungicide tolerance in several fungi (e.g., reference 171). *C. rosea* IK726 *abcG5* deletion mutants have increased sensitivity to *F. graminearum* culture filtrates, to ZEA, and even to several chemical

fungicides based on iprodione and mefenoxam. In contrast, strains complemented with functional *abcG5* regained tolerance to ZEA and the fungicides mentioned (172). Results from confrontation assays further indicated a role of ABCG5 in the secretion of toxic secondary metabolites by *C. rosea* that might give this transporter an important active role in mycoparasitic or biocontrol interactions (172). Deletion of *abcG29* resulted in mutants with reduced biocontrol ability toward *B. cinerea* and *F. graminearum* and with low H_2O_2 tolerance, although they were not affected in their sensitivity to ZEA, fungicides, or osmotic stress (173).

In contrast, *T. atroviride* Taabc2, which belongs to subfamily G group V as well (13), has an important role in tolerance to toxins and fungicides (174). Accordingly, the *Taabc2* gene is upregulated in the presence of pathogen-secreted metabolites, specific mycotoxins, and some fungicides (benomyl and dicloran), and under conditions that stimulate the production of antagonism-related secondary metabolites and CWDEs in *Trichoderma* spp. *Taabc2* deletion mutants show increased susceptibility to inhibitory compounds either secreted by plant pathogenic fungi or *Trichoderma* itself and reduced biocontrol activity, suggesting that Taabc2 also has a role in avoiding autointoxication of the mycoparasite by endogenous compounds with inhibitory action that are secreted during antagonism (174).

The *C. rosea* IK726 genome encodes 86 ABC transporters and 620 MFS transporters, which are among the highest numbers found in any fungi sequenced today (13). The observed expansions in the ABC subfamilies B group III and subfamily G group I and V probably reflect the lifestyle of *C. rosea* as a mycoparasite of toxin-producing fungi. The gene expansion in ABC transporter subfamilies in *C. rosea* differs, however, from that identified in the *T. virens* genome (that has a larger set of ABC transporters than *T. atroviride* with an expansion in subfamily C), indicating different lifestyles of mycoparasitic *Trichoderma* spp. and *C. rosea* (13).

A mycoparasite must also protect itself against its own hydrolytic enzymes secreted during the antagonistic interaction. However, only limited information is available about the mechanisms involved. In *T.* cf. *harzianum*, the cell wall-bound protein Qid74 has been shown to play a role in cell protection, and mutants with a disrupted *qid74* gene are more sensitive to lytic enzymes than the wild type (175). In addition, Qid74 is involved in adherence to hydrophobic surfaces, a function similar to hydrophobins. A high number of hydrophobic cell wall proteins are encoded in *T. atroviride* and *T. virens* mycoparasites. and the *qid74* gene is present in their genomes as well (28, 107). However, it has been found in only very few other fungi and is missing in *C. rosea* (M. Karlsson, unpublished data). In contrast to the high number of hydrophobins (up to 11 genes) in *Trichoderma* spp. (176, 177), only three hydrophobins are encoded in the *C. rosea* genome. Deletion of the *Hyd1* and *Hyd3* genes results in increased growth rate, which in turn leads to enhanced *in vitro* antagonism and biocontrol (16).

Epl-1, a protein of the cerato-platanin family, has been characterized in detail in *T. atroviride* and *T.* cf. *harzianum*. *T. atroviride* Epl-1 self-assembles at air/water interfaces and forms protein layers on surfaces including chitin, thereby increasing their polarity (178). In *T.* cf. *harzianum*, Epl-1 is predominantly present in the cell wall of the mycoparasite under confrontation conditions and has been suggested to have protection function or act as a recognition molecule to identify its own and/or host hyphae while avoiding self-degradation (179).

Carbohydrate-binding proteins could be involved in cell wall protection as well, as suggested by Gruber and Seidl-Seiboth (107). They could bind to short oligosaccharides and influence the induction of CWDEs or even bind to chitin and mask and protect it from degradation as reported for fungal-plant interactions (180).

The Role of Oxidative Stress in the Mycoparasitic Interaction

Fungal NADPH oxidase enzymes, responsible for the catalytic one-electron transfer of oxygen to generate superoxide or hydrogen peroxide (H_2O_2), play an important role in the development of fungal sexual structures and appressoria (181), as well as in ROS production during defense against other fungi. Overexpression of *nox1* in *T.* cf. *harzianum* resulted in elevated ROS formation during direct contact with *P. ultimum*, accompanied by an increased expression of protease, cellulase and chitinase genes (182).

Exposure of fungal spores or mycelia to H_2O_2 promotes oxidative stress (183). *T.* cf. *harzianum* ETS 323 secretes an L-amino oxidase (Th-LAAO) during mycoparasitism that is responsible for H_2O_2 production (184). Th-LAAO is a flavoprotein that catalyzes the oxidative deamination of an L-amino acid substrate into the corresponding α-oxoacid together with production of H_2O_2 and ammonia. Monomeric Th-LAAO induces hyphal lysis and apoptotic cell death in *R. solani*, whereas it has a stimulatory effect on hyphal density and sporulation of *T.* cf. *harzianum* itself. The antagonistic activity of Th-LAAO seems to be closely interrelated with its interaction with *R. solani* cell wall

proteins and their subsequent structural alterations (184, 185).

Interestingly, the *C. rosea* genome contains a high number of genes encoding members of two H_2O_2-producing enzyme classes, the aryl-alcohol oxidase and glucose oxidase, whereas *Trichoderma* spp. genomes only encode few respective enzymes (13). In addition, a high number of group B-III-type ABC transporters are encoded in the *C. rosea* genome including a homologue of *M. oryzae* Abc3 that is involved in resistance against ROS (186). Deletion of the ABC transporter gene *abcG29* in *C. rosea* resulted in delayed conidial germination in the presence of H_2O_2, which implies a function of ABCG29 in oxidative stress resistance (173). A possible functional connection between the high gene numbers of H_2O_2-producing enzymes and ABC transporters putatively involved in oxidative stress protection in *C. rosea* remains to be investigated. A novel strategy for oxidative stress protection found in *C. rosea* involves the secreted serine protease PrC that acts upon nematode cuticles to produce degradation products that protect against oxidative stress by scavenging ROS (187).

CONCLUSIONS

Mycoparasitism is a quite prevalent fungal lifestyle, and *Trichoderma* spp. and *C. rosea* share the ability for necrotrophic mycoparasitism as part of their broad environmental opportunism. From a comparative genomics perspective, the mycoparasitic lifestyle results in common features such as high numbers of genes encoding carbohydrate-active enzymes, proteases, membrane transporters, and proteins involved in biosynthesis of secondary metabolites. However, there are also several differences in the expansion of gene families of these well-studied fungal mycoparasites, indicating ecological differences. The low number of genes encoding chitin and glucan catabolic enzymes in *C. rosea*, compared with the expanded gene sets in *T. atroviride* and *T. virens*, suggests that degradation of the cell wall of the fungal prey is an important feature of mycoparasitism in *Trichoderma* spp. but not in *C. rosea*.

Recent genome analyses of additional hypocrealean mycoparasites confirmed that the biosynthesis of peptaibiotic secondary metabolites is a characteristic trait of these fungi. In addition, toxin-like substances play crucial roles in the specialized mycoparasite *E. weberi*. The dependence of *T. virens* on toxic metabolites (e.g., gliotoxin) that poison the fungal prey is reflected in several unique adaptations, i.e., high numbers of secondary metabolism-associated genes, of multidrug resistance-associated ABC transporters involved in toxin export, and of killer toxin-like chitinases that presumably permeabilize the cell wall of the fungal prey to facilitate toxin entry. In the case of *C. rosea*, the unprecedented numbers of ABC and MFS transporters involved in drug resistance emphasize the importance of toxin tolerance in this species.

Another observation is that many mycoparasitism-related genes appear to share certain characteristics not present in genes involved in endogenous functions, such as high interspecific copy number variation and specific expression during fungal-fungal interactions (e.g., killer toxin-like chitinases, pleiotropic drug resistance transporters, hydrophobins, and reducing-type PKSs). Parallels with the effector concept in plant-pathogen interactions may be drawn where the coevolutionary arms race between the pathogen and the plant host results in effectors/effector targets that exhibit similar characteristics.

Although the data available so far allow first insights into the multitude of genes and biological processes contributing to the mycoparasitic interaction, many questions remain open such as how the fungal prey is sensed and recognized by the mycoparasite and how biotic and abiotic interactions affect the mycoparasitic activity, issues that are important for the efficient, reliable, and tailor-made application of mycoparasitic fungi as biocontrol agents. Our knowledge concerning mycoparasites' genomics so far is based on a single genome (individual) per species. An important future topic will be to study within-species variation that will address the long-standing axiom of strain variation of biocontrol-relevant mycoparasitic traits. Recent advances also point out the importance of assessing and delineating the ecological niche(s) and life histories of mycoparasites to better interpret data emerging from comparative genomics and to allow highly targeted application of respective strains.

The emergence of genomic and large-scale transcriptomic and proteomic data and their comparative analyses beyond the genus boundaries can offer further important clues to our understanding of the molecular basis of mycoparasitism. In addition, transcriptomic and proteomic studies examining the reactions of both interacting fungi, the mycoparasite and its fungal prey, will provide further insights into mycoparasitism-related processes such as attack, counterattack, and defense contributing to this pathosystem. Several processes essential for the mycoparasitic fungus-fungus interaction such as host sensing and signaling, transcriptional reprogramming, and secretion of effectors, enzymes, and secondary metabolites are likely to be

shared with pathogens of plants and animals. On the other hand, however, sensing and signaling events as well as mycoparasitic responses depend on the host fungus showing that the mycoparasitic interaction is complex and fine-tuned. Therefore, more studies characterizing gene functions are essential to complement the basis provided by genomics.

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Fungi: Technology and Natural Products

IX

The Fungal Kingdom
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Fungal Enzymes and Yeasts for Conversion of Plant Biomass to Bioenergy and High-Value Products

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Lene Lange

THE ROLE OF FUNGI IN CONVERSION OF PLANT BIOMASS

The Increasing Importance of Fungi in Biotechnology

Fungi are now widely used in industrial biotechnology, for example, as production hosts for technical and food and feed processing enzymes, as gene donors for such enzymes, as production hosts for organic acids and cholesterol-lowering drugs (the statins), and as starter cultures and probiotics (1). Around half of the industrial enzymes used globally are of fungal origin; the other half are of bacterial origin. However, this balance is now moving toward the use of more enzymes from a wider spectrum of families of the fungal kingdom. There are several reasons for this. Fungal enzymes are efficient, compatible, and suitable for industrial processing: they have sufficient protein stability to give the enzyme products an acceptable shelf life; they provide customer solutions, meet regulatory approval demands, and fulfill end user needs.

The driver for this increased importance of fungi and fungal enzymes is the development of the new bioeconomy, where crop residues, industrial by-products, and organic waste streams are used as a basis for producing more bio-based products, obtaining more from biological resources, and wasting less (see Fig. 1). The potential is huge. Currently, there is a global loss of 30 to 50% (2) of all agricultural production, when summing up losses through the value chain from field to end user. Because of this, it is predicted that fungal enzymes will be vital for moving from a fossil-based to a renewables-based world economy, where upgrade of lignocellulosic biomass leads to production of not only biofuels, but also bio-based materials and bio-based chemicals, as well as new and healthier feed and food ingredients (3, 4).

Development toward a bioeconomy places fungi and mycology in a central position in relation to mitigating climate change; improving resource efficiency; replacing fuels, chemicals, and materials based on fossil resources; and contributing to feeding a growing global population. The next generation of products from biomass will utilize the intact structural complexity of the fresh biomass for health-promoting food and feed ingredients.

Fungal Enzymes, the Choice for Biomass Conversion: For Bioenergy and Beyond

Fungi have three roles in making value from plant biomass:

- As gene donors for large-scale production of enzymes
- As industrial production hosts for biomass-degrading enzymes and metabolites
- As producers of biorefinery end products (e.g., bioethanol, organic acids, biochemicals, or single-cell proteins) resulting from their growth on sugars from the enzymatic conversion of plant biomass

One of the most significant lifestyle characteristics of filamentous fungi is that they secrete enzymes. Secretion occurs primarily from the tip cells of the growing hyphae (5, 6). In nature, a large proportion of the secreted enzymes break down the substrate (e.g., plant tissue) that the fungus invades. In natural habitats, such

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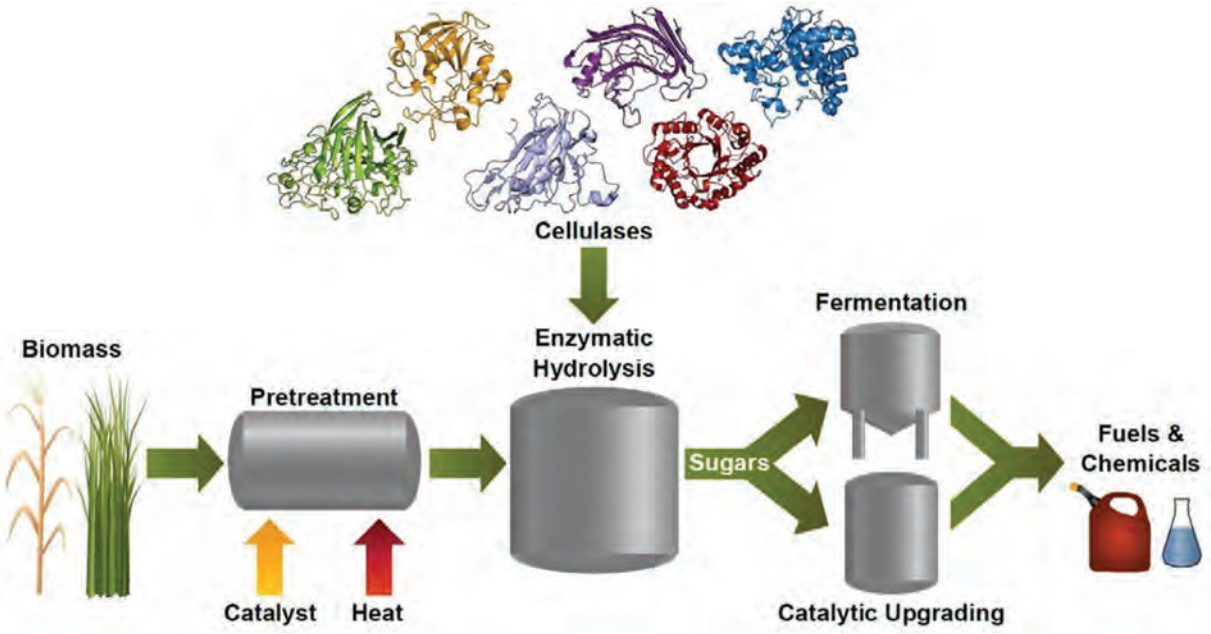


Figure 1 A schematic overview of a biorefinery. The product portfolio from biorefineries is not only fuels and chemicals but also includes higher-value products such as food and feed ingredients, cosmetics, skin care, and new functional biomaterials; it is also expected that many types of biorefineries will be developed for improved resource efficiency: the yellow (straw, stover, and wood chips), the green (fresh grass, clover, leaves), the blue (seaweed and fish bycatch and waste), the gray (agroindustrial side streams), and a biorefinery for upgrade of household waste and sludge (the brown biorefinery). Adapted from reference 16 with permission.

enzymatic biocatalysis of the substrate results in a conversion of the plant biomass into smaller building blocks suitable for hyphae to absorb for further growth of the fungus.

Through evolution, the growth of filamentous fungi has been adapted for efficiency in substrate utilization. The main adaptive traits are that (i) secreted fungal enzymes have a complement of activities for breaking down the substrate polymers, (ii) the enzymes are also stable outside the fungal cell, and (iii) the enzymes are available and are actively degrading the substrate at the time when the fungal hyphae have reached the area into which the enzymes have diffused. This allows the fungus to take up nutrients quickly after the degradation products have become available. Enzymatic breakdown thus primarily benefits the growth of the fungus itself rather than merely making substrate available for competitors such as bacteria or other fungi.

Almost all commercialized enzymes for industrial purposes (for example, in the detergent, textile, juice, paper, or baking industries) are enzymes (with a signal peptide for secretion) that in nature are secreted from the fungal cell which produces them. The reason for this is that secreted enzymes in general are more stable and significantly cheaper to produce because they can be recovered directly from large-scale fermentation broth without the necessity of opening the fungal cells.

Secreted, stable proteins suitable for large-scale, cost-efficient production can match the cost structure and price window of the industrial processes in question. Such proteins fulfill the primary industrial requirements, namely, high enzyme yields, easy recovery from fermentation broth, and good protein stability—all in all, giving lasting performance and end user benefits.

Overview of Secreted Enzymes from Filamentous Fungi

In the fungal kingdom a large spectrum of secreted enzymes has evolved to predigest organic structures into smaller molecules suitable for uptake by the fungal cell. Through evolution, fungal enzymes have been developed to break down almost all organic substrates available on earth (see the CAZy database [7]). The greatest diversity of enzyme activities described is related to the breakdown of plant cell wall structures. The CAZy database is described on its homepage as "a specialist

database, dedicated to the display and analysis of genomic, structural and biochemical information on Carbohydrate-Active Enzymes (CAZymes)." The CAZy database includes well-curated information about the following types of enzymes derived from all parts of the biological kingdoms: glycoside hydrolases (GHs) (hydrolysis and/or rearrangement of glycoside bonds), glycosyl transferases (formation of glycosidic bonds), polysaccharide lyases (nonhydrolytic cleavage of glycosidic bonds), carbohydrate esterases (hydrolysis of carbohydrate esters), and auxiliary activities (AAs) (redox enzymes that act in conjunction with CAZymes) (www.CAZy.org). In nature, fungal enzymes have been described that belong to all of these five groupings (GHs, glycosyl transferases, carbohydrate esterases, and AAs). Efficient conversion of biomass involves enzymes of all these major groups of carbohydrate active enzymes; GH cellulases and hemicellulases play the biggest role.

Biomass Conversion Enzymes Found Throughout the Fungal Kingdom and in Many Habitats

Genes encoding secreted enzymes are found in all divisions of the fungal kingdom. Based on the fungal enzymes included in the CAZy database, the richest portfolio of enzymes are in the *Ascomycota*, followed by the *Basidiomycota*, and lastly, the *Zygomycota*, whose members primarily produce enzymes suitable for releasing sugar molecules from already partially degraded products. Preliminary studies of the *Chytridiomycota* and *Neocallimastigomycota* document that at least selected species of these early-lineage fungi also have a wide portfolio of lignocellulosic polymer-degrading enzymes (8).

The most complex organic structures in plants and fungi are their cell walls. A huge number of different types of enzymes are needed for total breakdown of the plant cell walls of both monocots and dicots. Fungi specializing in degrading plant biomass in nature thus have a very broad portfolio of plant cell wall-degrading enzymes. Analysis and clustering of a broad spectrum of genome sequences of secretome enzymes from *Ascomycetes*, *Basidiomycetes*, and *Zygomycetes* gave a surprising result (9, 10): the clustering into being a good lignocellulose degrader or not was a more prominent trait than the fungal phyla to which the enzyme-producing fungi belonged. Thus, the phenotypic characteristic of the enzyme secretome portfolio overruled the taxonomic position.

Plant cell wall-degrading enzymes can be found in a vast spectrum of fungal habitats and life forms (Table 1). The most studied are white rot, brown rot, and soft rot fungi, as well as saprophytic fungi on dead herbaceous and wooden plant materials (11); interesting enzymes are also found from extremophiles (fungi growing in extreme environments) (12).

Table 1 A selection of well-studied lignocellulose degraders across the fungal kingdom

Fungal phyla	Fungal species	Fungal order	Physiology and habitat
Basidiomycota	*Phaenerochaete chrysosporium*	*Polyporales*	White rot
	Schizophyllum commune	*Agaricales*	Putative white rot
	Postia placenta	*Polyporales*	Brown rot
	Gloeophyllum trabeum	*Gloeophylales*	Brown rot
	Ganoderma applanatum	*Polyporales*	Heart rot
	Termitomyces spp.	*Agaricales*	Termite symbiont
	Leucoagaricus gongylophorus	*Agaricales*	Leaf cutter ant symbiont
Ascomycota	*Thermoascus aurantiacus*	*Eurotiales*	Saprotrophic
	Penicillium chrysogenum	*Eurotiales*	Saprotrophic
	Talaromyces leycettanus	*Eurotiales*	Saprotrophic
	Aspergillus niger	*Eurotiales*	Saprotrophic
	Myceliophthora thermophilae	*Sordariales*	Thermophilic, saprotrophic
	Neurospora crassa	*Sordariales*	Saprotrophic
	Melanocarpus albomyces	*Sordariales*	Saprotrophic
	Trichoderma harzianum	*Hypocreales*	Saprotrophic
	Fusarium oxysporum	*Hypocreales*	Necrotrophic
	Podospora		Coprophilic
Zygomycota	*Mucor circinelloides*	*Mucorales*	Saprotrophic
	Rhizopus stolonifera	*Mucorales*	
Chytridiomycota	*Rhizophlyctis rosea*	*Spizellomycetales*	Saprotrophic, soil
Neocallimastigomycota	*Piromyces communis*	*Neocallimastigomycetales*	Saprotrophic, rumen symbiont

Plant cell wall-degrading enzymes are also produced by plant pathogenic fungi (5) and by ectomyccorhizal fungi (13). Another biomass conversion habitat is that of coprophilic fungi, which grow on animal dung. Very prolific cellulase producers include *Podospora* spp. (14, 15). Coprophilic cellulase producers also typically secrete a range of bioactive metabolites and are found to be a better source of antimicrobial metabolites than most other cellulose-producing fungi: for coprophiles, substrate becomes available all at once, and the limiting factor for gaining access to the substrate is to outcompete other microbes present in the dung materials. In contrast, competition for access to substrate is not the primary parameter for plant pathogens. The infection process is very complex and depends primarily on breaking host resistance barriers.

PLANT CELL WALL-DEGRADING ENZYMES FROM FUNGI

The Recalcitrance of Lignocellulose and How To Overcome It

Lignocellulose is a highly complex and varying polymeric structure. It is the basic structure and major component of plant cell walls and has been developed through evolution to resist both biotic and abiotic stress, such as microbial pathogens and damage from wind and weather (16). Lignocellulose is composed of four major components: cellulose (20 to 50%), hemicellulose (15 to 35%), lignin (10 to 30%) (16), and pectin (often a rather low percentage). The lignocellulosic structure is highly recalcitrant to degradation, due to close packing of very complex, interlinked components. The branched hemicellulose coats the cellulose microfibers, the lignin encapsulates the polysaccharide components (cellulose, hemicellulose, and pectin) like a matrix around all polymeric structures, and the crystalline cellulose fibers themselves form a very recalcitrant structure, adding to inaccessibility and robustness. Due to this structural complexity and the interwoven, closely packed components, enzymes cannot readily access the plant cell wall components. Therefore, prior to industrial enzyme hydrolysis, a chemical/physical pretreatment is performed, which breaks down the lignocellulose structure sufficiently to allow enzymatic degradation.

Attempts have been made to develop more gentle pretreatment processes, for example, by using biocatalysis. The cellobiohydrolase enzyme family GH7 and laccases have been suggested for this purpose (17, 18) along with the use of family GH45-like proteins named expansins (19, 20), swollenins (21), and loosenins (16); lactic acid bacteria fermentation can also be used (16). Such gentler pretreatment could cut down energy costs and result in a hydrolyzed biomass that is more suitable as a base for upgrading, and it would not have the negative consequences of harsh pretreatments (e.g., artifacts, relocated lignin, and antinutritional factors).

Nature's biomass conversion processes have inspired research into the development of gentler pretreatment procedures. For instance, several *Ascomycetes* and *Basidiomycetes* can grow directly on and in a beech tree trunk; *Hypoxylon fragiforme* and *Schizophyllum commune* attack freshly fallen branches and trunks of deciduous trees, and *Fomes fomentarius* and *Ganoderma applanatum* cause heart rot on deciduous trees such as beech (22). Apparently, no pretreatment (physical or chemical) takes place. The fungi establish and grow, breaking down lignocellulose to fulfill their requirement for carbon. Time is obviously a factor, and changes over time in environmental conditions (humidity, temperature, light) play a role. However, molecules including the expansins, swollenins, and loosenins (16, 19–21) most likely also play a role. In addition, as yet undescribed synergies between chemical (reactive oxygen, photons, or metals) and biocatalytic reactions are probably involved. Interestingly, pretreatment is not required when aquatic biomass is used as biorefinery feedstock, because aquatic biomass has no or very little lignin in the cell walls.

Enzymatic Decomposition of Lignocellulose

Lignocellulose

Collective action among several enzymes is required to break down the complicated lignocellulosic structure. The most important group of enzymes for these activities is glycohydrolases, GH enzymes which cleave glycoside linkages (7). Such linkages are dominant in both cellulose and hemicellulose. Furthermore, polysaccharide esterases (carbohydrate esterases in the CAZy database [7]) assist the hydrolysis of GH enzymes by removing the methyl, acetyl, and phenolic esters. Polysaccharide lyases may also play a role in depolymerization. Several types of fungi, from a broad variety of habitats and taxonomic groupings, have all the enzymes needed for complete breakdown of lignocellulosic plant cell wall structures (see Table 1, above, and Fig. 3, below) (5, 16, 23–29).

Cellulose and cellulases

Depolymerization of cellulose fibers requires the following enzymatic activities: an endoglucanase acting on

cellulose (EC 3.2.1.4), two types of cellobiohydrolase (exoglucanases), one of which acts on the reducing end of the polymer (EC 3.2.1.177) and the other on the nonreducing end (EC 3.2.1.96), one β-glucosidase which cleaves the dimeric cellobiose to monomer C6 sugar/glucose (EC 3.2.1.21 β-glucosidase), plus the action of the lytic polysaccharide monooxygenases (LPMOs) (see Fig. 2A).

The occurrence of several β-1,4 endoglucanase-active enzymes (EC 3.2.1.4, of GH5, GH9, GH45) in a fungus is interpreted as reflecting a robust degrader whose enzymes contribute to improved fitness. This is because as a result, the fungus has built-in flexibility and can produce the enzymes most suitable for several types of environmental conditions (humidity, temperature, pH, etc.) (9).

Cellulases are divided into processive and nonprocessive; endoglucanases (EC 3.2.1.4) belong to the latter, and the cellobiohydrolases, GH6 and GH7, to the former. Furthermore, the hydrolytic activity can belong to the inverting (one-step) or the retaining (two-step) process (16). Among the active enzymes of the EC 3.2.1.4 class, GH45 is the only one of the inverting type.

Another prominent feature of cellulases in both nature and under industrial conditions relates to whether they are closely associated with the substrate, not just through interaction at the active site but also with a carbohydrate binding domain (CBM). The CBM can be located at either the N- or the C-terminal end (or in rare cases both) (30). CBMs can affect the efficacy and speed of enzyme activity; fungal CBMs primarily belong to the CBM1 family. Furthermore, whether the linker region that spans the catalytic domain and CBM is short or long also affects the behavior of the enzyme (27). CBM and linker modifications are such powerful tools for the design of enzymes and enzyme processes that they have been the basis of several patents and research and development (R&D) business strategies.

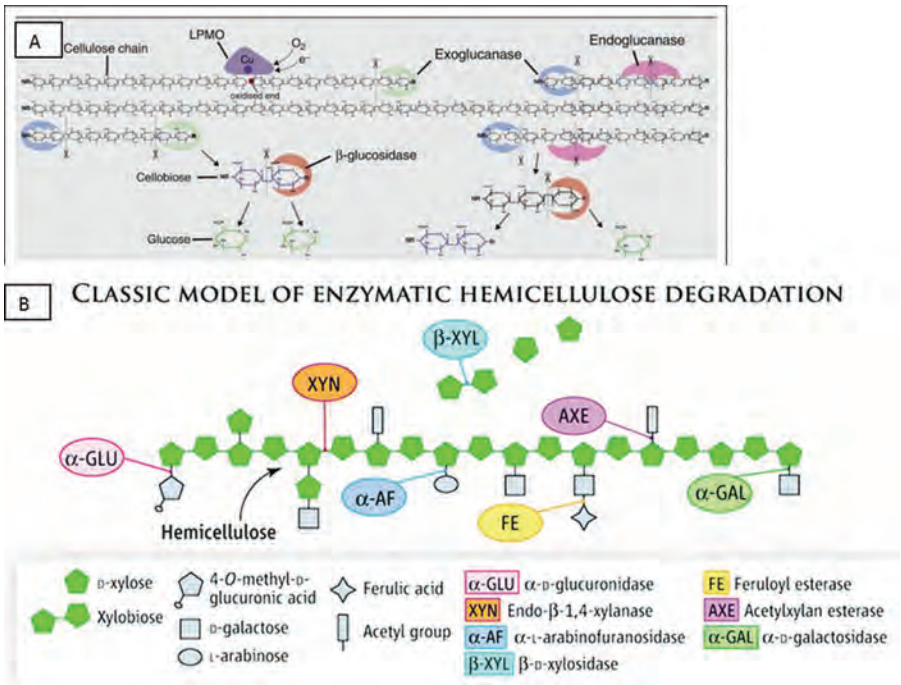


Figure 2 (**A**) Enzymatic breakdown of cellulose polymer includes several glycohydrolases (at least one endoglucanase, at least two cellobiohydrolases, reducing end and non-reducing-end active, and at least one β-glucosidase). Further, the activity of a lytic polysaccharide monooxygenase acts in synergy with the endoglucanase in breaking down the crystallinity of the cellulose polymer. Adapted from reference 24 with permission. (**B**) Hemicellulose is a very complex, highly branched and substituted polymer. The figure shows seven types of sugar components and lists the seven types of enzymes needed to break the linkages to such sugar moieties. However, enzyme hydrolysis of lignocellulose may not need the presence of all these seven hemicellulases because most of the standard pretreatment procedures will lead to the breakdown of several of the hemicellulose bonds. Adapted from reference 37 with permission.



Figure 3 "Fungal Hall of Fame" illustrating the five most important players in industrial lignocellulose biorefinery processing and in research. (**A**) *Aspergillus oryzae*, along with (**B**) *Aspergillus niger* and *Aspergillus nidulans*, are the most widely used monocomponent enzyme production organisms. (**C**) *Trichoderma reesei* is included due to its exceptional secretion capacity; it is the preferred production host for enzyme blends specifically designed for efficient biomass conversion. (**D**) *Saccharomyces cerevisiae* is the organism of choice for production of ethanol from the biomass conversion-derived sugar platform. *Pichia pastoris* is the expression host most often used for producing laboratory-scale volumes of newly discovered enzymes to facilitate characterization and evaluation of the new enzymes for industrial potentials. (**E**) *Myceliophthora thermophilae*, along with another thermophilic fungus, *Thermoascus aurantiacus*, represents alternatives to production of enzymes by species of *Aspergillus*. Credits: (A) Courtesy of Reinhard Wilting, Novozymes A/S; (B) from Read ND, *in Electron Microscopy of Plant Pathogens* (Mendgen K, Lesemann D-E, ed), Springer-Verlag, Berlin, Germany, 1991, with permission; (C) U.S. Department of Energy Office of Science (http://www.jgi.doe.gov/sequencing/why/Treesei.html); (D) Sciencephoto.com; (E) courtesy of Ronald de Vries, CBS-KNAW, Fungal Biodiversity Centre, The Netherlands.

Hemicellulose and hemicellulases

The depolymerization of heavily branched and substituted hemicellulose structures also requires an entire complement of enzymes. At least one kind of endoxylanase (e.g., GH10 or GH11) is needed (7, 31) to break the xylan backbone. The endoxylanase activity (EC 3.2.1.8) is assisted by a range of other hemicellulases, usually by synergistic activity among the following types of enzymes: acetyl xylan esterase, arabinofuranosidase, and ferulic acid esterase (32–37) (Fig. 2B).

The reason why many of the hemicellulase enzymes have not been included in commercialized enzyme blends for lignocellulose decomposition is that many of the linkages cleaved by such enzymes are already broken during the pretreatment processing.

Lignin

Lignin is a very different type of polymeric substance compared to cellulose and hemicellulose, because it is a heterogeneous alkyl-aromatic polymer. Lignin degradation by white rot basidiomycetous fungi has been more studied than any other type of fungi (38). The most prominent types of enzymes active in lignin degradation are laccases and peroxidases (both types are oxidative enzymes) (17, 39–41). However, such enzymes do not react independently, and mediators are

needed. Brown rot fungi such as *Coniophora puteana* and *Serpula lacrymans* do not have prominent lignin-degrading enzymes, but such fungi do modify (through the action of specialized reactive metabolites) the lignin surface structure, thus freeing the cellulosic components from the lignin. The modified lignin structure remains for years in nature without much further degradation. Research on lignin degradation has led to many new efforts in studying not only the classical brown rot and white rot fungi, but also many other fungal species in many habitats. From such new studies it appears that throughout the range of lignin-degrading fungi, there exists a continuum of biomass degradation regimes, with varying degrees of similarity to white rot and brown rot fungi (38).

A recent development in understanding the fungal decomposition of lignocellulosic structures is the discovery of a new type of enzymatic activity, which has a function significantly different from all of the biomass conversion enzymes mentioned above. The new kind of enzyme activity was revealed by applying enzyme discovery approaches that are independent of sequence or function similarity to known enzymes. Two approaches were used: (i) use of culture broth fractionation followed by estimation of lignocellulose decomposition potential of the various fractions and (ii) use of transposon-assisted signal trapping (TAST) screening, a technique discussed later in this article (42). Both techniques demonstrated that a significant and novel type of enzyme, hitherto not associated with cellulose breakdown, was present in the most active fractions and was highly expressed and secreted in all fungi that degraded lignocellulose (43).

The enzyme responsible for such activity was first described as a glycohydrolase and was assigned to the protein family GH61 (10, 44). Further studies showed that this new GH61 type was widespread in the fungal kingdom, typically present in high numbers in the genome, and was not a GH enzyme but a monooxygenase (45, 46). As a consequence, such members of GH61 were recategorized as AA enzymes, were categorized as AA9, and were named LPMOs. Since the first article describing AA9 was published, knowledge of the LPMOs has grown explosively (47–49). Now several types of LPMOs are described under the following classifications: AA9 (45), AA10 (50) (bacterial, primarily chitin degrading), AA11 (51, 52), and AA13 (53). Furthermore, LPMO activity has been described on substrates including starch (53), hemicellulose (54), and keratin (55). An interesting feature of genes encoding fungal LPMOs is that they are often found in very high numbers in the fungal genome (51).

How Can Nature's Effective Fungal Lignocellulose Degraders Be Recognized?

Fungal genome sequencing has shown that genes encoding cellulose- and hemicellulose-degrading enzymes are found in a very broad spectrum of fungi. However, many of such fungi may not necessarily functionally be effective biomass degraders, even though they have the spectrum of genes of relevance for lignocellulose decomposition.

The most efficient fungal lignocellulose degraders can often be recognized up-front by knowing their physiology and ecology: Does the species in nature always grow in habitats where biomass decomposition takes place? Is it a strong competitor on such substrates? Classical mycological expertise, such as knowledge about fungal habitats and substrate associations, can provide answers to such questions and is particularly relevant and valuable for developing more efficient biomass conversion processes. At the DNA level, genome sequencing gives information about whether the species in question has the full complement of cellulase and hemicellulase genes. However, to predict the potential of a fungal species, we need to know not just which families of enzyme genes are present on the genome but also for which enzyme function the genes code. Assigning genes to a given enzyme family is not enough to identify its function because most enzyme protein families include several types of function (7). Since gene sequences which are very distantly related may share the same function, blast searches and alignment are not sufficient to predict the function. Based on this analysis and reasoning, efforts were made to develop a new sequence analysis method which could more accurately predict function from sequence.

As a result, a new non-alignment-based sequence analysis method was invented (56); it was later developed into a versatile and fast peptide pattern recognition (PPR) technology platform (9, 10, 51, 57). PPR can identify short peptides which are conserved among enzymes with shared function. Based on this, groups of proteins with the same enzyme function can be identified. With this approach, enzyme discoveries can be made, mining sequence databases efficiently and predicting enzyme function directly from sequence.

Different Mechanisms in the Fungal Breakdown of Cellulose

The enzymatic breakdown of cellulose in nature follows three or four models (16, 58–61): (i) fungal enzymatic breakdown where enzymes with different functionalities (endoglucanase, CBHs, betaglucosidase, and LPMO) are produced by separate genes, which results in individual

enzymes acting in synergy; (ii) cellulose breakdown, such as by brown rot fungi, where redox-acting enzymes give rise to ions needed for Fenton chemistry reactions to occur, which are capable even of breaking down the crystalline cellulose; and (iii) the bacterial model, where the genes needed for cellulose breakdown are placed sequentially in one reading frame and the proteins are placed in one huge molecular structure, the cellulosome, which processes the entire cascade of enzymatic reactions needed for breaking down cellulose; (iv) recently, a new category has been recognized, where enzyme hydrolysis is achieved by multimodular enzymes (*viz.*, enzymes with multicatalytic domains [59, 62]). Such multimodular enzymes have been described and are expected to be found in more fungi degrading plant biomass. Recent studies also suggest that in some habitats, such as anaerobic environments, most of the secreted xylanolitic and cellulolytic enzymes are found as free enzymes and are not organized in cellulosomes; they are, however, to a large extent encoded by multimodular genes (Wilkens C, Busk P, Pilgaard B, Kirkegaard R, Albertsen M, Zhang W-J, Nielsen P, Lange L, in press).

Interestingly, fungal enzymatic breakdown of cellulose (category [i], above) follows very closely the bacterial enzymatic breakdown of chitin (*viz.*, endo-acting chitinase plus reducing- and nonreducing-end, exo-acting chitinases (50). Moreover, bacterial decomposition of chitin also involves LPMO activity—the bacterial AA10 family (45). The resemblance of cellulose decomposition by fungi to chitin decomposition by bacteria is remarkable in evolutionary terms.

Recently, a new mode of action of fungal enzymatic degradation of the proteinaceous polymer keratin has been described (64). Fungal breakdown of alpha keratin can involve three types of proteases: endo-acting, exo-acting, and oligopeptide-acting (65); this pattern is thus also similar to fungal cellulose decomposition. Furthermore, keratin-degrading fungi (including the dermatophytes) also have LPMO genes (of type AA11) in their genomes (51). Such new knowledge could be applied to develop new technology for degrading chicken feathers and pig bristles, which so far are almost totally unexploited protein-rich waste.

YEAST FERMENTATION: FROM SUGAR PLATFORM TO BIOETHANOL

Fungal Yeasts: The System of Choice for Producing Bioethanol

Yeast cultures have been used to produce ethanol since ancient times. Even after centuries of industrial and academic practice, development, and research, *Saccharomyces cerevisiae* is still the biological production system of choice. This yeast is easy to control in industrial settings, and the robustness of wild-type *S. cerevisiae* against the cell toxicity of ethanol is acceptable. Increased ethanol tolerance can also be achieved through strain development, by mutation, or by genetic modification.

The primary focus of most of the research to improve the efficiency of ethanol fermentation from the sugar platform has been on using a larger range of the sugars of the biomass or, more precisely, to improve yeast fermentation to exploit not only C6 sugars (cellulose-derived glucose) but also C5 sugars (hemicellulose-derived xylose). Some progress has been achieved (see below). Further progress may be achieved by following one of three approaches: (i) make bioethanol only from the cellulose C6 sugars and reserve the hemicellulose C5 monomer and oligo sugar for making higher-value products such as food or feed ingredients; (ii) improve the efficiency of ethanol-fermenting yeast strains for fermenting both C5 and C6 sugars to ethanol; or (iii) develop new fermentation systems that produce a new generation of biofuels (66).

Higher-value products from hemicellulose (C5) sugars can be achieved, for example, by producing food and feed ingredients, based on the complexity (branching and substitutions) of the native hemicellulose polymers. These products can be refined to even higher value by making food and feed ingredients that have health-promoting effects such as prebiotic stimulation of the healthy gut flora of humans and nonruminant animals (67, 68). The resulting yeast biomass can also be upgraded to health-promoting ingredients, beta-glucans, nutraceuticals, or even drugs, based on the specialized fungal cell wall structure properties.

Development of Improved Yeast Fermentation Strains

In nature, yeast is primarily specialized to ferment C6 sugars. Until now, no wild-type microbe (yeast or bacteria) has been found which readily fulfills industrial demands such as efficient fermentation (high yields, rapid processing) of C5 sugars to ethanol and high tolerance to ethanol content in the fermentation broth.

Interesting attempts have been made to develop the bacterium *Thermoanaerobacter mathranii* (typical habitat is hot springs in North Atlantic areas) into an efficient ethanol production strain using both C5 and C6 sugars. High temperature tolerance makes recovery of the ethanol possible by skimming from the top of

the fermentation culture, so the critical ethanol concentration detrimental to the production strain is never reached (69). So far, a similar approach for a thermophile yeast has not been developed.

Improved yeast strains have been developed by several small companies, for example, Terranol. An *S. cerevisiae* strain that efficiently ferments C5 sugars and yeast strains that also grow on and utilize glycerol were developed (70, 71). For such progress, the key genes to control were Gut1p (glycerol kinase) and Gut2p (mitochondrial glycerol-3-phosphate dehydrogenase) originating from *S. cerevisiae* (72, 73). The next goal is to develop yeast strains capable of producing advanced biofuel (see below), as well as relevant chemical building blocks. The latter pave the way for renewable production of plant biomass-based chemicals, substituting for the current production of fossil-based chemicals. In the years to come the fuel- and chemical-producing organisms will be fungal, probably refined strains of *S. cerevisiae* (and in certain cases also strains of filamentous fungi), in versatile roles producing increased value from biomass. The core technology for this is enzymatic hydrolysis of the plant cell wall biomass, releasing free sugars from the plant polymers; this is followed by fermentation of fungi on the plant biomass-derived sugar platform.

BIOREFINERIES

Advanced Biofuels, Integrated Biorefineries, and On-Site Production of Enzymes

The terminology around different types of biofuels is very confusing. There are not only first- and second-generation biofuel, but also third- and fourth-generation, and a clearly defined term is needed. The European Biofuel Technology Platform (http://biofuelstp.eu/) has suggested and defined "advanced biofuels" as the term of choice. Advanced biofuels are defined as (i) being produced from lignocellulosic feedstock (not directly from starch, lipid, or sugar), (ii) having low CO_2 emission or high greenhouse gas reduction, and (iii) achieving zero or low indirect land use change impact.

Most of the advanced biofuels involve fungal enzymatic breakdown of biomass and fungal production of the advanced biofuel, but alternative systems are also being developed which do not involve fungi or fungal enzymes. These include advanced biofuels from algae or the use of physical and chemical processes, e.g., bio-dimethyl ether, biosynthetic natural gas, and bio-oil (75).

Integrated biorefineries (76) are not yet sufficiently cost-effective to be commercially viable. However, improved consolidated efficiency may be achieved by developing fungal strains which can produce the enzymes needed for biomass conversion and also produce ethanol. Alternatively, fermentation conditions may be created which allow two types of fungi to work in parallel under conditions controlled by changes in pH or another flexible parameter.

Enzymes may be produced on-site (77) directly in the production plant. The required enzymes are, for example, produced on a side stream of the feedstock, and the entire content of the enzyme-producing seed tank is then added to the main tank to start the enzymatic biomass hydrolysis. Intellectual property and technology protection issues, restrictions on the use of genetically modified organism (GMO) strains in industrial-scale biorefineries, or suboptimized on-site enzyme blends for efficient biomass conversion have so far blocked such development. However, when biorefineries are developed in which several higher-value products are produced from the feedstock, efficiency may be measured in terms different from the current emphasis on quantification of free sugars.

DISCOVERY AND PRODUCTION OF FUNGAL ENZYMES FOR BIOMASS CONVERSION

Production of Fungal Enzymes by Heterologous Expression in Fungal Production Hosts

The most classic and impressive example of development of an enzyme production strain is the early industrial use of cellulases. The history of the development of *Trichoderma* into a production host for cellulases (16) highlights the application of mycological knowledge to solve an industrial problem and to contribute to more sustainable agroindustrial processing.

In general, the fungi chosen as expression and production hosts have primarily been filamentous ascomycetous species such as *Trichoderma reesei* (78), *Aspergillus oryzae* (79, 80), *Aspergillus nidulans* (81), and *Aspergillus niger* (82, 83). Efficient production strains of *Fusarium venenatum* have also been developed (84). Further, attempts have been made to develop *Thermoascus aurantiacus* into a production host for heterologously expressed genes of thermotolerant enzyme proteins (85). Yeasts have also been developed as production hosts, e.g., *S. cerevisiae* (86) and *Pichia* spp. (87), but in yeast the yield is in general significantly lower than what can be achieved with filamen-

tous fungi. Yeasts are inferior with regard to protein secretion capabilities compared to filamentous fungi. This is an inherent characteristic of the yeast unicellular life form, with budding propagation and growth, because yeasts would not benefit from secreted enzymes that diffuse into the surrounding substrate. Figure 3 displays the five most prominent fungal species used in biomass conversion.

The company Dyadic has taken an interesting approach to the design of production hosts. Production strains of *Myceliophthora thermophilum* were developed in which the background of its native enzymes provided added value to the enzyme product; for instance, the native *M. thermophilum* cellulases boost the effect of the cellulases of a GMO construct (*M. thermophilum* with inserted cellulase genes, derived from other organisms) (88).

The latest significantly new type of application of GMO constructs of *T. reesei* is that it is used as a production strain for producing not just one enzyme but blends of recombinantly produced plant biomass-converting enzymes (e.g., Novozymes CellicCTec3: http://www.bioenergy.novozymes.com/en/cellulosic-ethanol/CellicCTec3/Pages/default.aspx) (90–92). Several genes are inserted into the genome, and the fermentation is developed to give an optimized blend, designed for efficient biomass conversion. It is noteworthy that for blends, most of the cellulase genes of *Trichoderma* have been replaced with superior cell wall-degrading enzymes from other fungi. Thus, the unique capability of *Trichoderma* to produce and secrete enzymes, and not the qualities of the *Trichoderma* enzymes, make this fungus the system of choice for biomass blend production.

Yeast production of industrial enzymes in bulk is still only barely commercially viable at a large scale, even though fermentation technologies leading to high yeast cell density (93) have to a certain degree compensated for the lower yield. However, at the laboratory scale, heterologous expression systems based on *Pichia* spp. and *S. cerevisiae* are commonly used as the system of choice for production and characterization of novel eukaryote proteins. The low level of glycosylation that takes place in these expression host systems can, however, be a drawback (87).

Interestingly, *Ascomycetes* spp., for instance, *Trichoderma* spp. and *Aspergillus* spp., also function well as production hosts for a broad spectrum of enzyme genes derived from other parts of the fungal kingdom (from species of *Basidiomycota*, *Zygomycota*, and even *Chytridiomycota*). However, production, for example, of basidiomycete genes in ascomycetous hosts may still result in lower yields compared to ascomyceteous genes (94). Codon-optimized synthetic genes may solve or at least minimize this problem.

Plant enzymes that also are of importance for biomass conversion have proven difficult to produce in filamentous fungi. Interestingly, the only effective expression of plant enzymes in fungi has been in yeasts and not in filamentous species (95, 96). Studies have been conducted to understand why successful expression of plant enzymes in filamentous fungi is blocked. Hamann and Lange found that filamentous fungal expression strains of *A. oryzae* misread the sequence of the plant enzyme gene as splicing sites, leading to production of fractionated mRNA and therefore to the lack of production of functional plant proteins (97).

Details about how to construct the most efficient fungal production hosts and which level of yield is achievable are primarily found in patent literature (giving a basis for the patent claims) or are more commonly kept as company-confidential. To be competitive in the industrial biotech industry, it is essential to have a very cost-efficient and cost-competitive enzyme production and enzyme recovery system, and the relevant details are considered strictly confidential.

An important part of developing a production host system is to modify the production strain by deletion of genes. Modifications may include abolishing end product inhibition and knocking out protease genes, which may break down the enzyme protein produced, and finally also to knock out the background of secreted enzymes which may interfere with the final enzyme product.

Another important issue is to have the fungal production strain approved by regulatory authorities, preferentially as a GRAS (generally recognized as safe) organism (98). This is needed for approval of the production plant and production process and is of special importance if food-grade enzymes are to be produced.

Fungi as Gene Donors: Discovery of New Industrially Relevant Fungal Enzymes

The technologies used for the discovery of new and improved fungal (and bacterial) enzymes have undergone significant development since the start of large-scale commercialization of microbial enzymes. The knowledge and experience gained during the different stages of industrial biotech are also relevant to future development of the bioeconomy. Several chapters of the new segment of industrial biotech (the circular bioeconomy, where local bioresources such as crop residues and agroindustrial side streams are upgraded to higher value in nearby biorefineries) lie ahead: big biorefineries will

be supplemented by smaller, decentralized and specialized use of bioprocessing; the need for new blends of enzymes for conversion of new types of complex biomass will grow. The use of microbial consortia may, in special instances, supplement the current pure culture fermentations, and new types of enzymes will be needed and new mechanisms of biocatalysis synergy may be discovered. This development will involve the increased use of a diversity of fungal (and bacterial) enzymes.

The inspiration for such development will be through a technology push, where new methods pave the way for new solutions. Likewise, studies of nature's own biomass conversion will also inspire and drive discoveries. Combining the two could prove to be a powerful strategy. Such developments inherently imply that discovery technologies should also be modified and developed to function efficiently at the metagenomics and meta-transcriptomics level, including the unculturable part of the microbiomes (99).

Enzyme Discovery from Nature

From the start of the industrial use of enzymes, the goal of discovery of fungal enzymes has been to find fungal isolates with a high level of activity on relevant defined or complex substrates. The enzyme protein should be stable and have pH and temperature profiles that match industrial process conditions. By this approach the discovery is based on experimental laboratory evaluation of the fungal strain and its enzymes, handling as one sample the entire multienzyme culture broth, with enzyme concentrations given by the fermentation physiology. Such screening and evaluation conditions favor the discovery of enzymes produced in high concentrations by their wild-type fungus and preferentially detect enzymes with good stability and high specific activity. However, with this approach it is easy to overlook enzymes for which no assays have been developed or cases in which several enzymes are needed from more than one organism (100).

A large spectrum of enzyme products relevant for industrial biotechnology was developed in the 1960s, 1970s, and first half of the 1980s based on such an enzyme discovery strategy. The strategy tapped into the microbial diversity available from culture collections and from the pool of naturally occurring microorganisms. Two examples are amylases for the detergent industry (bacterial, e.g., from *Bacillus* spp.) and cellulases for the textile industry (fungal, e.g., from *Trichoderma* spp. [101]). These examples relate to two very different industrial processes: (i) high pH and specific temperature stability and activity requirements and (ii) low pH and a broad spectrum of acceptable temperature activity profiles.

Exploitation of the early cellulase enzyme discoveries depended on more than the activity of the enzyme. The native fungal producer needed to grow well under submerged, aerated, fermentation conditions and to produce a good yield of the enzyme protein under such conditions. Basically, developing enzyme manufacturing systems with low production and recovery costs laid the foundation for industrial biotechnology. A realistic value proposition could be presented, which could convince industrial decision makers that biological processing could compete with chemical processing in cost, as well as in robustness and performance. The new biological processing also meant changing to a sustainable process technology, with less polluting chemicals in the wastewater and less energy use for high pressure and high temperature used in chemical processing. The emerging development of such competitive biological processing that was able to replace chemical processing (102) opened the way for setting environmental requirements to obtain regulatory approval of production facilities. This in turn meant that new environmental standards could be set, drawing up limits for acceptable negative impacts on the environment. The third type of driver for the development of gentle and competitive biological processing was to make improved use of the raw material, i.e., to obtain more product from the same raw material. Fungal cellulases were developed for treatment of, for example, fruit pulp after initial juice processing. A cellulase treatment could result in significantly higher yield of juice (and wine) simply by breaking down more of the plant cell walls to allow more juice to be extracted (103).

Next, the textile industry needed an enzyme for stone washing of jeans (104) and enzymes for the detergent industry for more gentle, color-protecting washing powder (105). Both of these commercial targets facilitated investments in significant R&D efforts to find new and interesting fungal cellulases. An important discovery was the GH45 protein, produced by the thermophilic fungus *Humicola insolens* (*Ascomycota*, *Sordariales*) (106). The enzymes of *H. insolens* are active at rather high pH (pH 8) and stable at elevated temperatures, with the maximum at ca. 45°C (104, 105).

During this period the foundation was laid for developing biocatalysis to replace many of the previously chemistry-based processes. But in the following period, the 1980s and 1990s, huge steps were taken to build the basis for understanding the complexity of plant cell wall-degrading enzymes. This knowledge was later used to develop the blend of cellulases needed for converting lignocellulosic biomass into biofuel (89, 106).

Monocomponent Enzymes Produced by Fungal GMO Production Strains

Analysis of the efficiency of enzyme production, e.g., productivity per tank year or enzyme yield per liter of fungal culture broth produced, revealed that much of the production capacity of the fungus and of the fermentation tanks was used to produce enzymes which did not add direct value for the customers (107).

Around the same time, in the 1980s, GMO technology was in its infancy, primarily focusing on making GMO constructs of plant cultivars (108) and in *Escherichia coli* (109, 110). From this research it became apparent that both prokaryote and eukaryote cells were able to produce much higher yields of single proteins than had been observed in wild-type strains. The use of a range of different promoters and of methods for selection of the highest-yielding transformants also inspired further development of microbial industrial biotechnology.

In 1988 the first GMO strain of an industrial enzyme was produced. It was a recombinant, monocomponent lipase enzyme for the detergent industry, expressed in *A. oryzae* (111). It was produced in Novo's facility in Japan because Danish legislation for industrial handling of GMO strains was not yet developed (112). This success inspired a new strategy among the leading industrial biotechnology companies (Novo and Danisco in Denmark, DSM in The Netherlands, and Genencore in the United States). Dialogue with, e.g., the Danish Environmental Protection Agency authorities (http://eng.mst.dk/) was very constructive, and both industry and authorities wanted strict regulation. Many years later, this proved to be a very wise decision. The strict requirements and regulatory conditions for approval of GMO strains and GMO production facilities, processes, and products laid the foundation for public acceptance of this new technology. The production vehicle was GMO-based but biologically confined to the fermentation plant, with no living GMO present in the end product and therefore no GMO organism reaching the end user. A new era of industrial biotechnology had begun.

Discovery and Optimization of Genes for Production of Monocomponent Enzymes

The initial step was finding a fungus with enzyme activities of interest and the necessary protein stability under industrially relevant pH and temperature conditions. This step was the same as for discovery of the classical non-GMO enzymes. But after this initial step, the entire discovery process was radically different. The first attempts to separate the wanted enzyme out of the complexity of the culture broth were tedious, costly, and slow; even if successful, discovery of the specific gene which could be inserted in the production host was far from easy. After a promising fungal isolate with interesting enzyme characteristics had been identified, the obvious next step was to focus on the genes, not the protein, and a strategy was developed as follows: (i) isolate the mRNA (which as a pool represents all expressed proteins), (ii) use this pool of mRNA to identify the cDNA of interest, and then (iii) insert this cDNA into the (heterologue expression) production host. By using cDNA, the uncertainty about whether the heterologue expression host could cope with the splicing of a foreign gene was circumvented, because cDNA has no introns. Only the coding part of the reading frame is transformed into the production host (100).

The next step was to efficiently develop (and screen) high-quality cDNA libraries to identify specific enzyme activities of interest. This process was developed to perfection in industry. During this period cDNA library screening became the single most used molecular technology in enzyme discovery, and it has resulted in the vast majority of new fungal enzyme discoveries commercialized over a period of 10 to 20 years. To date, a major proportion of commercially available fungal industrial enzymes have been found by cDNA screening technology (100).

The principles of high-throughput fungal cDNA technology screening (114) are as follows. (i) Prepare fungal biomass which has been exposed to the substrate components of relevance for the enzymes of interest. This is usually done by growing the selected fungal strain on its native substrate or on a well-defined medium containing the substrate of relevance for the enzyme activity of interest. (ii) Prepare total RNA followed by a polyA (tail) column separation of the mRNA fraction of the total RNA. (iii) Make the cDNA on the basis of the mRNA. (iv) Insert the cDNA in an *E. coli* library for propagation of the cDNA. (v) Insert the cDNA in a yeast expression host. For this, an *S. cerevisiae* strain was chosen with very limited background enzyme activity, and a substrate-induced promoter was included which only opened for gene expression if galactose was present in the growth media. The resulting yeast cDNA library was then screened for activity on multiple substrates by replica plating. Stock cultures and plates were maintained without promoter-inducing galactose to avoid fatigue of the selected transformants with promoter turned on all time. Such series of transformants were screened by measuring the activity obtained in small-scale fermentation, leading to selection of the transformants with the highest yield. The result of such

cDNA screening (100) was a yeast clone harboring the gene of interest that was known to be expressed and secreted; the gene found was full length and gave rise to a secreted, functional protein with the relevant activity.

The patent strategy for enzyme discovery and for product and process technology protection improved dramatically, along with development of monocomponent enzymes, GMO production strains and production processes, and value-added, end-product uses. It was of particular interest to extend the product patent coverage, which provided a basis for and resulted in protection significantly broader than just for the enzyme found. As a consequence, with patent protection as a driver, a series of new technologies were developed. These included approaches for the identification of conserved areas of the genes of interest to construct primers for PCR screening, thereby allowing for the discovery of nature's variants of the same gene (115). Patent examples based on such discoveries were a basis for broader claims of variation in sequence identity and for patent claims covering immunological recognition. One of the largest such efforts is Novozyme's GH45 patent claiming nature's variants of the GH45 gene across all fungal kingdom phyla (116).

Protein Engineering to Improve Enzymes for Conversion of Plant Materials

Three significant activities led to the development of molecular biotechnology R&D as a prominent field in its own right: (i) discovery of monocomponent protein backbones, (ii) sequencing of the enzyme proteins discovered becoming accessible and affordable, and (iii) elucidation of three-dimensional enzyme protein structures. Protein engineering (PE) became a scientific discipline and an important part of industrial biotech R&D. Both random mutagenesis and targeted mutagenesis were applied with a high level of success. The efficiency of particular enzymes with robust activity could be significantly improved through random or targeted mutation schemes to modify the optimum pH and temperature to match industrial needs. Another target of protein engineering was to change enzyme stability and robustness toward chemical and biochemical process agents. Generally, PE developed into a complex operation in which many rounds of different technologies eventually were merged together in one process, named artificial evolution. Along with such methodological progress, the use of high-throughput, automated laboratory screening by robotics was developed to build on not just smartness in thinking but also number-crunching power as a basis for selection of the superior enzyme activity (117–119).

A dedicated nonevolutionary technology—shuffling technology (*viz.*, cutting the gene to pieces, shuffling the gene parts, and reassembling them into new sequences)—was also developed as part of the artificial evolution. This was the basis for improving function to a level hitherto not achievable through mutations and selection (120, 121).

Protein engineering R&D efforts became so successful that for a period it appeared that discovery of new wild-type enzymes from nature's diversity might not be needed in the future. The diversity needed to satisfy industrial needs and end user demands was predicted to be met by engineering already known protein DNA backbones. However, it became apparent that finding activities radically different from known enzymes was most efficiently achieved by screening wild-type microorganisms and then using PE to optimize on such a backbone.

Next-Generation Sequencing Revolutionized Enzyme Discovery

The next-generation sequencing technologies paved the way for a new generation of microbial discovery strategies (63, 122). Initially, the technology was a shortcut to the integrated use of sequencing of, e.g., PCR bands identified in PCR screening searching for more variants of a lead enzyme gene, and for dynamic and fast sequencing of the new genes discovered by, e.g., cDNA screening to allow fast and efficient priority patenting. In short, the new-generation sequencing technology became a game changer: sequencing was much faster, prices dropped, and sequencing capacity, availability, and accessibility increased. Whole-genome sequencing became part of a standard enzyme protein discovery procedure which generated new sequences and mined sequences which had been placed in the public domain by other scientists with insufficient knowledge of the enzymes coded for by such deposited genes. Efficient enzyme discovery was now *de facto* tripartite: (i) discovery of new wild-type enzyme backbones from nature's diversity, (ii) discovery from man-made diversity (PE variant and mutagenesis libraries), and (iii) discovery by *in silico* mining of deposited sequences. The three approaches could strategically be placed sequentially: identifying interesting organisms by determining interesting habitats and by activity profiling such organisms, sequencing the genome of the selected organisms, and mining the new genomes along with other related genomes and genes and selecting the most promising genes for expression for further characterization of the enzyme protein. If gene expression was successful and the protein had promising activities,

automated screening of variant libraries would generate fast and improved versions of the new enzyme.

The limiting factor and pitfall for such new, sequence-based enzyme discovery is 2-fold. There may be a rather high risk of unsuccessful expression of the selected gene, and the limited number of available enzyme activity assays fails to precisely detect the desired novel type of activity.

Two new types of technologies, TAST and PPR, were developed to optimize exploitation of the overwhelming amount of sequence data available from new-generation sequencing of both eukaryote and prokaryote organisms (42, 74). Both technologies aimed at optimizing the possibilities of finding novel and improved types of enzyme activities from enzymes which are also expressed as functional proteins in nature, therefore with optimized perspectives for good expression in industrial production hosts.

The basic principle of TAST (42) is reflected in the construction of the transposon: a β-lactamase gene without its signal peptide is inserted (together with other marker genes) into the transposon. When a new gene is inserted in the *E. coli* screening host along with the transposon, the transformant cell, when screened on ampicillin-holding plates, will survive only if the gene has a signal peptide. This is because the β-lactamase protein is transported across the cell membrane and therefore correctly folded only if the β-lactamase gene on the transposon is fused with a signal peptide (from the new enzyme gene), making the β-lactamase active and thus protecting the transformant cell against ampicillin. Surprisingly, TAST screening could be used for discovery of novel genes and proteins from all groups of prokaryotes and eukaryotes even though the determining screening criterion is transportation over the *E. coli* cytoplasmic membrane. Enzymes with totally novel enzyme sequences and enzymes for which we did not have any assays available could now be found by TAST (42, 97).

A convincing example of the significance of the new TAST technology was the discovery of a new type of enzyme (GH61), which was found as being expressed in high numbers in almost all TAST screening projects performed with cellulose- and lignocellulose-degrading fungi (43). GH61 (now known as LPMO, AA9) is now a standard component of commercialized plant biomass-degrading enzyme blends.

TAST, however, was not an easy technology to exploit. Most laboratories which tried it did not succeed. New and improved methods were needed for finding secreted enzymes. Further, a need arose for making it possible to find also the sequence commonality among even distantly related proteins sharing the same function. Based on this, a new protein discovery technology, PPR, was invented.

PPR is a non-alignment-based, sequence analysis method which identifies conserved n-mer peptides and enables the assembling of a group of proteins sharing more than a set number of conserved peptides from a given list (56). Such PPR groupings have been validated to correlate to the enzyme function (10) and can be used (through application of additional PPR technology platform software programs) to predict function directly from sequence (9). PPR is faster and more efficient than other methods in mining even very large data sets. PPR can also be used to efficiently mine huge databases of metagenomic as well as metatranscriptomic sequences.

PPR enables discoveries of functions where screening assays have not been developed and facilitates the discovery of sequences of very distantly related genes with low shared sequence identity but the same function. It is currently superior to other sequencing analysis methodologies because it predicts the function directly from the sequence, which is especially valuable if discoveries are sought from microorganisms or habitats where annotated genome sequencing is not available. PPR can also be used efficiently to find new discoveries from poorly annotated sequence databases and to find more members of described families than have been found before (leading to *in silico* expansion of protein families). PPR brings order out of chaos.

The low cost of synthetic genes—codon optimized to the expression system of choice (for fungal genes, e.g., *Pichia pastoris*)—has made *in silico* discoveries (combined with PPR sequence analysis) a shortcut to the discovery of novel enzymes, e.g., for efficient conversion of new types of feedstocks.

FUTURE PERSPECTIVES: UPGRADING MORE ORGANIC FEEDSTOCKS TO EVEN HIGHER VALUE AND NEW BUSINESS MODELS

Exploitation of enzymes for the bioeconomy upgrade of complex organic residues, by-products, and waste streams has ushered in a new era of biological production. Earlier, monocomponent enzymes produced in GMO production hosts were commercialized following the business model of one gene, one enzyme, one product. However, the new production technology and business model for biomass conversion enzymes builds on several different genes transformed into one and the same production strain (for example, *T. reesei*), leading to production of a designed composite blend of enzymes (89, 90).

The next steps are to design enzyme blends for processing of more types of biomass, such as new types of plant feedstocks and agroindustrial by-products, biomass of fungi (e.g., vinasse, the spent microbial biomass from biorefineries), and of macroalgae (primarily brown and red), as well as to develop upscaled methods for valorization of organic fractions of household waste and sludge. The focus will broaden from being confined to the breakdown to monomer sugars to include recovery of selected structural components of the biomass for higher-value products (e.g., health-promoting prebiotics).

Systems for "on-site production" of enzymes may also be developed, for example, by growing fungi that produce blends of enzymes directly on the organic fraction of household waste and using this enzyme blend for hydrolysis and liquefaction of the organic part of the mixed waste stream. Development of hydrolysis and liquefaction processing of organic household waste has already been accomplished (see http://www.renescience.com/en). Similarly, fungal starter cultures could be developed for direct "on-farm" upgrade of crop residues (e.g., from vegetable production) to a protein-rich and bioaccessible animal feed.

Another future approach is to promote the use of fungal enzymes and fungal starter cultures in developing countries at the village level for upgrading organic waste to fuel, feed, and fertilizer. Such decentralized use of enzymatic biomass conversion could be promoted further if local scientists, mycologists, agronomists, and plant pathologists could more readily access the cultures and the knowledge of biomass conversion. A vision has been formulated for an open access biology (1) administered by international culture collections, where the essential strains and knowledge could be accessed. Strains earmarked for open access biology should be safe, efficient production hosts, gene donors, or starter cultures that are available in the public domain with freedom to operate royalty free.

MYCOLOGICAL PERSPECTIVES: FUNGAL SECRETOME, EVOLUTION, AND DYNAMICS

The enzyme composition of the secretome of filamentous fungi provides a basis for efficient biomass conversion and valorization. More research into the evolution and dynamics of the fungal secretome, put in evolutionary perspective, is essential for understanding biomass conversion in nature and for developing biomass conversion even further. In nature, evolutionary pressure is on the secretome and on the enzyme functions, proteins, and genes, because increased fitness is achieved through efficient enzymatic breakdown of available organic materials (89, 113). The mechanisms for adaptation of fungal secretome composition and enzyme functions are gene copying, followed by gene loss or gene retention, similar to the process of speciation, but faster. Evidence is growing of the prevalence of an additional mechanism: horizontal gene transfer. This occurs in special habitats such as cow rumen and gut channels but is expected to be found more widely among fungal habitats, including other biomass conversion hot spots. Studies of such habitats can pave the way for interesting, new conceptual understandings of relevance for both mycology and applied use.

The fungal gardens of termites and leaf cutter ants can be seen as nature's prototype for the yellow and the green biorefineries (28). In the garden, fungal biomass is used to feed entire colonies. Similarly, new higher-value products derived from fungal biomass could be the next important new step in the bioeconomy for improving the health of both humans and livestock (68).

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Fungal Ligninolytic Enzymes and Their Applications

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INTRODUCTION

Lignin is an aromatic polymer with a heterogeneous structure. Varied, nonhydrolyzable linkages between subunits present a significant constraint to efficient exploitation of plant lignin. The different types of biomass and pretreatment used in biomass conversion processes result in a large variety in the molecular weight, structures, modifications, amount, and aqueous solubility of the lignin in the remaining product side streams. While polysaccharides are deconstructed biologically mostly by glycoside hydrolase and esterase hydrolysis, the ether and carbon-carbon bonds of lignin are attacked by fungal oxidative enzymes and small-molecule oxidative species, adding heterogeneity to the products.

Lignin is the second most abundant renewable biopolymer on Earth, after cellulose, and accounts for 26 to 32% and 20 to 25% of the dry weight in softwoods and hardwoods, respectively (1). In gramineous plants, lignin content varies across plant species and tissues, such as 15 to 21% of the dry weight in corn stover and 5 to 17% of the dry weight in wheat straw (2). Cross-linking of lignin to hemicellulose provides strength to the plant (3), aids in water transportation, and protects the plant cell from microbial attack (4). Lignin consists of three monolignols synthesized through the phenylpropanoid pathway: sinapyl, coniferyl, and *p*-coumaryl alcohol (5, 6). Lignin contains various C-C and C-O bonds, but the β-O-4 aryl ether linkage is most abundant (30 to 40% in softwood and 40 to 50% in hardwood) (7). The mix of woody components by tissue and species also may determine carbon-active enzyme activity (8). Recent analysis of genome content in metagenomes and diverse wood decay fungi has unearthed new candidate lignin-interacting enzymes (9–12).

Research has built a detailed picture of the biosynthesis and structure of lignin. Genetic modification of the lignin pathway in plant systems has enhanced the digestibility of cell walls due to (i) lower lignin content, (ii) increased access to cellulose and hemicellulose, and/or (iii) more easily digested bonds in lignin itself (13–15).

BIOMASS PRETREATMENT AND ITS EFFECT ON LIGNIN

Pretreatment broadly covers the thermochemical processes that open the structure of biomass so that it becomes susceptible to subsequent enzymatic hydrolysis of polysaccharides and enzymatic oxidative deconstruction. Until recently, pretreatment research focused on polysaccharide exposure, with little concern about lignin other than as a component for removal or alteration to avoid inhibition of enzymatic hydrolysis. Changes in the chemical structure of lignin due to pretreatment may make the resulting lignin less suitable for interaction with fungal enzymes, because it is then no longer a natural substrate. A diverse range of biomass pretreatments are used, and these generate differences in quantity and structure, as well as solubility of lignin breakdown products. The amount of lignin is also dependent on the feedstock substrate, and therefore the choice of pretreatment is also dependent on the ultimate product.

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PRETREATMENT METHODS

In alkaline pretreatments, strong bases (e.g., sodium, calcium, or ammonium hydroxide) are used to partially degrade (open) the (hemi-)cellulose structure, but they also result in structural changes in lignin (16, 17). Up to 60% of lignin can be solubilized and separated from cellulose by alkaline treatment. Under alkaline conditions the phenolic alkyl-aryl ethers in lignin are cleaved, resulting in a more soluble lignin (18).

Acid pretreatment is usually performed using dilute sulfuric acid (19, 20), though processes using hydrochloric acid are also used. This pretreatment removes the hemicellulose (21), but not lignin. However, it does alter the structure of lignin, cleaving β-O-4 ether linkages and generating a β-ketone or an aldehyde group on one lignin molecule and a free phenolic group on the other (22). Drawbacks of the process are condensation of lignin (23) and side products that reduce sugar to ethanol efficiency (e.g., furfural and hydroxymethylfurfural) (24).

Ionic liquids are salts that are in liquid form at room temperature and have a high dissolving capacity for lignocellulose (25). The advantage of using ionic liquids is that they largely leave the various polymers intact, resulting in more native substrates, including lignin, for subsequent enzymatic depolymerization (26, 27).

Steam explosion pretreatments are a short exposure of the feedstock to high temperature (190 to 270°C) and pressure (0.7 to 4.8 megapascal) followed by rapid pressure reduction, resulting in disruption of the fiber structure (28). Dilute acids are often used to facilitate the process (28). Lignin is partially removed or redistributed during the steam explosion, resulting in modified lignin (29), caused by cleavage of acid-labile linkages such as the predominant β-O-4 ether linkage (30).

Similar to steam explosion, liquid hot water is an autohydrolysis process that uses liquid water at high pressure and elevated temperatures (160 to 240°C) (31). Liquid hot water pretreatment results in cosolubilization of hemicellulose and lignin and in separation of this product stream from the solid cellulose-rich fraction. Solubilization of lignin together with hemicellulose is more efficient at lower temperatures (32). The treatment can result in an increase of the ratio of acid-insoluble to acid-soluble lignin due to an altered lignin structure (23).

In ammonia fiber explosion, the feedstock is exposed to liquid anhydrous ammonia at high pressure and moderate temperatures (60 to 100°C) followed by rapid pressure reduction, in a process similar to steam explosion (33–35). Rapid expansion of the ammonia gas results in swelling of the feedstock, which breaks lignin-hemicellulose linkages. However, ammonia fiber explosion does not remove lignin, but deposits it on the surface of the feedstock (34) and alters its structure significantly (36).

In ammonia recycle percolation, 5 to 15% aqueous ammonia is fed through a packed bed reactor containing the lignocellulose feedstock at temperatures between 140 and 210°C (37). This process differs from ammonia fiber explosion in that it removes 75 to 85% of lignin and solubilizes 50 to 60% of hemicellulose (38). In organosolv pretreatment, delignification is less pronounced compared to organosolv pulping. It is performed at high temperatures (100 to 150°C) using a variety of solvents that cause breakage of internal bonds of the lignin polymer (36). Lignin is removed in varying degrees depending on the solvent (39) and can be separated containing a high amount of aromatic rings (40).

Ozonolysis treatment of biomass efficiently removes lignin due to an electrophilic attack followed by a hydroxylation reaction of the aromatic ring (41). This results in a partial conversion of acid-insoluble to acid-soluble lignin, enabling the removal of a significant proportion of the lignin (42). While ozone also reacts with carbohydrates, these reactions are about 106 times slower than with lignin, resulting in specific lignin removal (41).

Supercritical fluid pretreatment uses materials that can be in either a gaseous or liquid state and are used under temperature and pressure conditions where both states coexist. They therefore can penetrate the crystalline structure of lignocellulose (43). No removal of lignin has been reported for this treatment.

LIGNIN-MODIFYING FUNGI

Basidiomycetous species are the only organisms able to efficiently mineralize lignin into CO_2. Lignin-modifying basidiomycetous species have been traditionally divided into two groups based on the visual appearance of the wood residue. White rot fungi (e.g., *Phanerochaete chrysosporium*, *Dichomitus squalens*, *Trametes versicolor*) degrade all the major polymers in plant cell wall including lignin. As a result, the decayed wood is characteristically white, and it includes fiber-like cellulose-enriched material (44). Litter-decomposing fungi are also able to cause white rot-type decay. Brown rot fungi (e.g., *Gloeophyllum trabeum*, *Postia placenta*) depolymerize cellulose in the early stage of wood decay, whereas lignin is only modified, e.g., by demethoxylation. The remaining brown wood residue has decreased strength (45, 46). However, a more nuanced classification of wood-rotting fungi has been suggested.

The more diverse mechanisms of wood decay have been revealed by analysis of the basidiomycete genomes, which reflect a spectrum of lifestyles between brown and white rots and ectomycorrhizal associations (47, 48). Wood-colonizing ascomycete fungal species such as *Xylaria* spp. have potential in mineralizing lignin, although to a lesser extent than wood-decaying basidiomycetes (49). Soft rot, which can attack the softer parts of the wood that have lower lignin content, is often a characteristic of the ascomycetes, though there are reports that soft rot characteristics are present in some Basidiomycota (48).

Brown rot fungi lack class II peroxidase enzymes, which encompass lignin-active AA2-type peroxidases. Phylogenetically, brown rot lifestyles have evolved at least five times from white rot lineages (48). Brown rot fungi have also lost many carbohydrate active enzymes (e.g., GH6, GH7, AA9) and oxidoreductases, enzymes that may be energetically costly to exploit lignin as a carbon source. Brown rot genomes have an expansion of both GH5 and GH28 gene families, which target cellulose and hemicellulose. Active fluidity between lifestyles is observable through phenotyping and genome characterization, reflected by pseudogenization of peroxidases in *Fistulina hepatica*, a relative of the white rot *Schizophyllum commune* (50).

Lignin decay takes place both enzymatically through the activity of enzymes and through nonenzymatic radical-based breakdown. The current paradigm of nonenzymatic rot activity is that fungi lower their environmental pH and secrete protective and iron-sequestering oxalate, other iron chelators, and enzymes that produce oxygen radicals (51). Hydroxyl and hydroperoxyl radicals generated by electron exchange between iron and hydrogen peroxide, as well as iron-reductant phenolates diffuse into the wood, continuing to reduce iron for reactivity with hydrogen peroxide, and both react with lignin elements which are then released (52). These diffusion reactions are a first foray into breakdown in both white rot and brown rot fungi, because decay of the plant cell wall takes place at a distance from the fungal hypha (53). Differences between lignin utilization (by white rot fungi) and lignin modification or repolymerization (by brown rot fungi) may be due to efficiency differences of nonenzymatic Fenton reaction-based wood penetration (52). The large size of many white rot lignin-active enzymes is thought to prevent access to portions of the wood (54).

Wood growth experiments (50, 52) have shown that enzyme production and activity on a single biomass type varies, which suggests that optimization of overall or specialized enzyme activity for industrial deconstruction may benefit from knowledge of the many new genomes of fungi being sequenced and phenotypically characterized (11, 48, 50, 55). Additionally, small molecule reactivity and Fenton chemistry intermediates can be investigated *in vitro* (56), leading to lignin byproducts and modifications that may be useful for extraction or lignin bioproduct manufacture. As with cellulosic enzyme mixtures, inclusion of different accessory enzymes and nonhydrolytic proteins (57–59), chemistries (52, 56), or timing of enzyme activity (60) may improve industrial manipulation of lignin. For instance, higher molecular weight products from lignin can be produced from laccase enzyme-producing fungi, while peroxidase activity is correlated with smaller molecular weight wood components (60).

STRUCTURE AND ACTIVITY OF LIGNIN-ACTING ENZYMES

Enzymes involved in lignin depolymerization include oxidative enzymes that catalyze nonspecific reactions (Table 1). Several of these enzymes are catalogued in the Carbohydrate-Active enZymes Database (CAZy; http://www.cazy.org/) as a family of auxiliary activities (AAs) (61).

Fungal class II heme-containing peroxidases, i.e., lignin peroxidases (LiP), manganese peroxidases (MnP), and versatile peroxidases (VP), classified in the AA2 family, are the key enzymes for lignin degradation (44). By using H_2O_2 as a cosubstrate, they catalyze oxidation of high-redox aromatic substrate molecules. Genes encoding lignin-acting peroxidases have been found exclusively in genomes of basidiomycete white rot fungi (47, 62, 63). The overall architecture of lignin-acting peroxidases from primary to tertiary structure is well conserved, including Ca^{2+}-binding sites, heme-pocket residues, and four disulfide bridges (64).

LiPs were the first heme peroxidases shown to be associated with ligninolysis (65, 66). LiP enzymes have been reported only from a few white rot fungal genera such as *Bjerkandera*, *Phanerochaete*, *Phlebia*, and *Trametes* (47, 62, 67). The redox active residue needed for LiP catalysis is a solvent-exposed tryptophan (Trp-171 in *P. chrysosporium* LiP H8 [68]), which facilitates the oxidation of lignin and other bulky aromatic substrates by long-range electron transfer to a heme cofactor (69, 70). A similar solvent-exposed tryptophan is conserved in VPs (71). LiPs and VPs are also distinguished from the other lignin-acting peroxidases by their high redox potential (1.4 to 1.5 V) that facilitates direct oxidation of nonphenolic lignin substructures (72). LiP catalyzes one-electron oxidation of a broad range of

Table 1 Fungal oxidoreductases involved in modification of lignin and lignin-derived compounds

Enzyme	CAZy family[a]	Systematic name/EC no.	Reaction	Characterized enzymes from fungi
Laccase	AA1_1	Benzenediol oxygen oxidoreductase/ 1.10.3.2	4 Benzenediol + O_2 ⇒ 4 benzosemiquinone + 2 H_2O	Widely distributed in ascomycetes and basidiomycetes
Manganese peroxidase (MnP)	AA2	Mn(II):hydrogen-peroxide oxidoreductase/1.11.1.13	2 Mn(II) + 2 H^+ + H_2O_2 ⇒ 2 Mn(III) + 2 H_2O	White rot wood and litter-decomposing fungi (e.g., *Dichomitus squalens*, *Agaricus bisporus*, *Agrocybe praecox*)
Lignin peroxidase (LiP)	AA2	(3,4-Dimethoxyphenyl)methanol: hydrogen-peroxide oxidoreductase/ 1.11.1.14	(3,4-Dimethoxyphenyl)methanol + H_2O_2 ⇒ 3,4-di-methoxybenzaldehyde + 2 H_2O	Some white rot fungi (e.g., *Phanerochaete chrysosporium*, *Phlebia radiata*, *Trametes versicolor*)
Versatile peroxidase (VP)	AA2	Reactive-Black-5:hydrogen-peroxide oxidoreductase/1.11.1.16	(1) Reactive Black 5 + H_2O_2 ⇒ oxidized Reactive Black 5 + 2 H_2O (2) Donor + H_2O_2 = oxidized donor + 2 H_2O	Only in some white rot species (e.g., *Pleurotus ostreatus*, *Bjerkandera adusta*)
Dye-decolorizing peroxidase (DyP)	na	Reactive-Blue-5:hydrogen-peroxide oxidoreductase/ 1.11.1.19	Reactive Blue 5 + 2 H_2O_2 ⇒ phthalate + 2,2′-disulfonyl azobenzene + 3-[(4-amino-6-chloro-1,3,5-triazin-2-yl)amino] benzenesulfonate + 2 H_2O	Common in ascomycete and white rot and litter-decomposing basidiomycetes (e.g., *Geotrichum candidum, B. adusta, Auricularia auricula-judae*)
Chloroperoxidase (CPO)	na	Chloride:hydrogen-peroxide oxidoreductase/1.11.1.10	RH + chloride + H_2O_2 ⇒ RCl + 2 H_2O	Litter-decomposing basidiomycetes (e.g., *Agaricus bisporus*)
Unspecific peroxygenase (UPO)	na	Substrate:hydrogen peroxide oxidoreductase (RH-hydroxylating or -epoxidizing)/1.11.2.1	RH + H_2O_2 ⇒ ROH + H_2O	Litter-decomposing basidiomycetes (e.g., *Agrocybe aegerita*)
Cellobiose hydrogenase (CDH)	AA3_1	Cellobiose:acceptor 1-oxidoreductase/ 1.1.99.18	Cellobiose + acceptor ⇒ cellobiono-1,5-lactone + reduced acceptor	Ascomycetes and basidiomycetes (e.g., *Myceliophtora thermophila*, *D. squalens*)
Aryl-alcohol oxidase (AAO)	AA3_2	Aryl-alcohol:oxygen oxidoreductase/ 1.1.3.7	Aromatic primary alcohol + O_2 ⇒ aromatic aldehyde + H_2O_2	White rot fungi (e.g., *Pleurotus eryngii*)
Glucose oxidase (GOX)	AA3_2	β-D-Glucose:oxygen 1-oxidoreductase/ 1.1.3.4	β-D-Glucose + O_2 ⇒ D-glucono-1,5-lactone + H_2O_2	Ascomycetes and basidiomycetes (e.g., *Aspergillus niger, P. chrysosporium*)
Methanol oxidase (MOX)	AA3_3	Alcohol:oxygen oxidoreductase/ 1.1.3.13	A primary alcohol + O_2 ⇒ an aldehyde + H_2O_2	Some ascomycetes and basidiomycetes (e.g., *Penicillium amagasakiense*, *P. chrysosporium*, *Gloeophyllum trabeum*)
Pyranose 2-oxidase (P2O)	AA3_4	Pyranose:oxygen 2-oxidoreductase/ 1.1.3.10	D-Glucose + O_2 ⇒ 2-dehydro-D-glucose + H_2O_2	Some basidiomycetes (e.g., *P. chrysosporium*, *Trametes ochracae*)
Vanillyl-alcohol oxidase (VAO)	AA4	Vanillyl alcohol:oxygen oxidoreductase/ 1.1.3.38	Vanillyl alcohol + O_2 ⇒ vanillin + H_2O_2	Ascomycetes, *Penicillium simplicissimum*
Glyoxal oxidase (GLX)	AA5_1	na/ 1.1.3.–	RCHO + O_2 + H_2O ⇒ RCOOH + H_2O_2	White rot and brown rot basidiomycete species (e.g., *P. chrysosporium*)
Galactose oxidase (GAO)	AA5_2	D-Galactose:oxygen 6-oxidoreductase/ 1.1.3.9	D-Galactose + O_2 ⇒ D-galacto-hexodialdose + H_2O_2	Some ascomycetes (e.g., *Fusarium graminearum*)
Benzoquinone reductase (BQR)	AA6	NADPH:*p*-benzoquinone oxidoreductase/1.6.5.6	NADPH + H^+ + *p*-benzoquinone ⇒ $NADP^+$ + hydroquinone	Some white rot and brown rot basidiomycetes (*P. chrysosporium*, *G. trabeum*)

[a]na, not available.

substrates such as phenols, aromatic amines, aromatic ethers, and polycyclic aromatics generating cation radicals (73, 74). This results in C_α-C_β cleavage, aromatic ring oxidation, and cleavage of the dimeric lignin model compounds (75, 76). One example of nonphenolic high-redox-potential aromatic substrates of LiP is veratryl alcohol (3,4-dimethoxybenzyl alcohol), which is converted to veratraldehyde by LiP catalysis (77, 78).

Like LiPs, VPs directly oxidize high-redox-potential aromatic compounds and recalcitrant dyes. These include compounds such as Reactive Black 5, which LiP can oxidize only in the presence of redox mediators (79, 80). VPs also possess Mn-oxidizing activity, combining catalytic properties of both LiP and MnP. The best-characterized VPs are from the white rot fungal genera *Pleurotus* and *Bjerkandera* (79, 81) (see Table 1). Interestingly, LiP of *Ceriporiopsis subvermispora* is able to directly oxidize both veratryl alcohol and Reactive Black 5, and its VP lacks the ability to oxidize Mn^{2+}. This suggests that *C. subvermispora* enzymes represent an intermediate evolutionary state between LiPs and VPs (82).

MnPs are the most prevalent lignin-acting enzymes in wood- and litter-decomposing white rot fungi. They specifically catalyze oxidation of Mn^{2+} to Mn^{3+}, which diffuses from the enzyme and oxidizes phenolic substrate. Mn-binding sites of MnPs consist of three acidic amino acid residues (Glu-35, Glu-39, and Asp-179 in *P. chrysosporium* MnP1) that hexacoordinate the Mn^{2+} ion. Mn^{3+} ions are chelated by dicarboxylic acids, e.g., oxalic acid, into a more stable form. This facilitates the diffusion of the chelated Mn^{3+} into the intact lignified secondary cell wall with micropores <6 nm in diameter, which are inaccessible by large enzymes (53, 83). However, due to their lower redox potential (1.0 to 1.2 V), MnPs cannot directly oxidize nonphenolic lignin structures (72). MnP activity on lignin is mediated by lipid peroxidation, whereby reactive intermediates, i.e., peroxyl radicals, are generated from lipids, resulting in the MnP-lipid system, which is powerful enough to attack bonds in nonphenolic lignin substructures (84–86). In addition, gene models encoding atypical MnP and VP enzymes have been reported (50, 62), based on conserved Mn-binding sites and a solvent-exposed tryptophan residue.

Two new superfamilies of secreted fungal peroxidases have been described, i.e., dye-decolorizing peroxidases (DyPs) and heme-thiolate peroxidases, which are involved in degradation or modification of lignin. DyPs are phylogenetically unrelated to LiPs, VPs, and MnPs. Numerous DyP-encoding gene models are present in the genomes of fungi, bacteria, and archaea (88), but only a few of the enzymes have been biochemically characterized (89). DyPs are interesting enzymes from a biotechnological standpoint, since they oxidize various dye compounds, carotenoids, and phenolics (90, 91). Also, a low-level oxidation of veratryl alcohol and nonphenolic lignin model compounds has been reported for DyPs (92, 93), whose redox potential (1.2 to 1.5 V) is comparable to even that of high-redox-potential LiPs and VPs (72). Recently, the first fungal DyP able to efficiently oxidize Mn^{2+} was characterized from the white rot basidiomycete *Pleurotus ostreatus* (94). However, the biological role of DyPs is still unclear.

The heme-thiolate peroxidase superfamily includes nonspecific peroxygenases (UPOs). UPOs are catalytically versatile enzymes that, unlike peroxidases, catalyze the addition of oxygen atoms into aromatic and aliphatic compounds (95, 96). While the physiological role of heme-thiolate peroxidases is unclear, their catalytic diversity indicates a putative function in conversion of lignin-related compounds. For instance, UPOs cleave nonphenolic β-O-4 lignin model dimers (97). UPOs have been biochemically characterized from the litter-decomposing fungi genera *Agrocybe*, *Coprinellus*, and *Marasmius* (98).

Laccases, in the CAZy family AA1_1, belong to the diverse multi-copper oxidase superfamily (99). Typically, laccases contain four copper atoms. Laccases catalyze four consecutive one-electron transfer reactions from various phenolic compounds, aromatic amines, and dye molecules (see Table 1) (100). The redox potential of fungal laccases is low (0.5 to 1.0 V), but in the presence of suitable low molecular weight redox mediator compounds such as 1-hydroxybenzotriazole and 2,2-azinobis(3-ethylbenz-thiazoline-6-sulfonate) their substrate spectrum is expanded to nonphenolic compounds and larger organic polymers (101). Laccase and multi-copper oxidases display a highly conserved three-dimensional structure with four substrate binding loops and four highly conserved signature sequences which contain amino acids that act as copper ligands. Phylogenetically, multi-copper oxidases can be divided into subfamilies including *sensu stricto* laccases and Fe^{2+}-oxidizing Fet3-like ferroxidases, the latter associated with pigment formation and putative ascorbate oxidases (102). Fungal laccases are thought to participate in lignocellulose decay, morphogenesis, plant pathogen interactions, and stress defense (100, 103). However, some lignin-degrading white rot fungi lack genes encoding *sensu stricto* laccases (e.g., *P. chrysosporium* and *Phanerochaete carnosa*).

Extracellular generation of H_2O_2 is important for catalysis by all heme peroxidases and for generation of

highly reactive hydroxyl radicals via Fenton chemistry (73, 104, 105). Various oxidases have been associated with biodegradation of wood and lignin as providers of extracellular H_2O_2. These include the glucose-methanol-choline oxidoreductase superfamily and copper radical oxidases. Other methods of H_2O_2 production, such as Mn(III)-mediated oxidation of fungal-secreted organic acids, also exist (106).

Glucose-methanol-choline superfamily members such as cellobiose dehydrogenase (CDH), glucose oxidase (GOX) (107), aryl-alcohol oxidase (AAO) (108), pyranose 2-oxidase (P2O) (109), and methanol oxidase (also known as ethanol/alcohol oxidase) (110, 111) are classified in AA3. All glucose-methanol-choline superfamily members share similar structural features and catalytic activity, oxidation of an alcohol moiety to the corresponding aldehyde. They are flavoproteins that contain a conserved flavin-adenine dinucleotide-binding domain (112, 113) and two conserved histidines (His-497 and His-541 in *Bjerkandera adusta* AAO) at the active site (114, 115). In P2O, methanol oxidase, and CDH, the second histidine is replaced by asparagine.

CDH has been classified in the CAZy subfamily AA3_1. CDH is a heme-containing flavocytochrome which oxidizes cellobiose and cello-oligosaccharides to the corresponding lactones using a wide spectrum of electron acceptors such as dioxygen, quinones, and phenoxy radicals (116–118). CDH enzymes have been suggested to generate hydroxyl radicals through Fenton chemistry, potentially oxidizing lignin substructures (116). Typically, white rot species possess only a single CDH-encoding gene (67).

GOX and AAO are structurally related and belong to the subfamily AA3_2. GOX oxidizes the hydroxyl group at the C1 position of sugars concomitantly producing H_2O_2, while AAO generates H_2O_2 by oxidizing aromatic primary alcohols to their corresponding aldehydes (114). AAO has been mostly studied in *Pleurotus* species such as *Pleurotus sajor-caju* (119), *Pleurotus eryngii* (108), and *P. ostreatus* (120) and in *B. adusta* (121). Redox cycling of aromatic fungal metabolites such as anisaldehyde (*Pleurotus* spp.) and 3-chloro-p-methoxybenzaldehyde (*B. adusta*) provides a continuous source of H_2O_2 to peroxidases (122–124). Copper radical oxidases such as glyoxal oxidase (GLX) have broad substrate specificity for the oxidation of simple aldehydes such as glyoxal and methylglyoxal to the corresponding carboxylic acids with the reduction of O_2 to H_2O_2 (125). GLX is a copper metalloenzyme containing an unusual active site free radical-coupled Cu similar to the structure in galactose oxidase (125). GLX is the most studied member of the copper radical oxidases and is physiologically linked to LiP in *P. chrysosporium*, making H_2O_2 to aid LiP activity (126).

Knowledge about conversion of lignin-derived monomeric intermediates, e.g., quinones, hydroquinones, and benzaldehydes, by intracellular enzymes is scarce. Benzoquinone reductases (CAZy AA6 family), vanillyl alcohol oxidases (CAZy AA4 family), cytochrome P450 mono-oxygenases, glutathione *S*-transferases, and *O*-methyl transferases have been implicated in fungal aromatic metabolism (127).

INDUSTRIAL APPLICATIONS OF LIGNIN-ACTING ENZYMES

While a major application of fungal lignin-acting enzymes is the depolymerization of lignin to facilitate the conversion of lignin to value-added products, other uses have been developed. Laccases are widely exploited due to their diverse substrate range, which can be even further extended using redox mediators, though mediator costs can be an obstacle for application (128, 129). Laccases are used for degradation of pollutants and xenobiotics (130–138) and for decolorization and degradation of chemical dyes commonly used in the textile industry (139–142). Other environmental applications involve the treatment of effluents (142–144), bleaching (145), and waste removal (146, 147) in the pulp and paper industry. Laccases polymerize catechol to polycatechol, a redox polymer used for production of chromatographic resins and thin films for biosensors (148). Laccase produces the polymer poly(1-naphthol) (149–151), found in wood composites, resins, fiber bonding, coatings, and adhesives (149).

Laccases have been used to produce a variety of pharmaceutical compounds, such as in the conversion of 4-methyl-3-hydroxyanthranillic acid to actinocin, an anticancer drug (152). It can also couple katarantine and vindoline to form vinblastine, which is used in leukemia treatment (129). Other pharmaceutical compounds produced using laccase are herbamycin derivatives and other aminoquinones (153, 154), oxidized catechins (155, 156), and hormone derivatives (157, 158).

In other applications, DeniLite, a fungal laccase, has been used to produce amine-derivatized platinum electrodes for detection of phenols, which have a very fast response time and long stability (159). Similarly, recombinant laccases are used for graphite-electrode-based phenol biosensors (160).

Lignin-related enzymes other than laccases are exploited in several applications (161). MnP from the white rot fungus *T. versicolor* has been explored for the bleaching of wood pulps (162), while a VP from

the white rot fungus *Pleurotus sapidus* has carotenoid-degrading activity (163). Transformation of a range of pharmaceutical compounds was reported with a VP from another white rot species, *B. adusta* (164). DyP from the litter-decomposing basidiomycete *Marasmius scorodonius* is able to degrade β-carotene (165), a role that has potential for the food industry.

APPLICATIONS OF LIGNIN AND LIGNIN PRODUCTS

Lignin applications and products use the highly versatile properties of lignin polymers and their functional groups, either alone or complexed with other molecules or templates (166). Its ability to scavenge radicals makes lignin a natural antioxidant (167). Biobased polymers like lignin are attractive alternatives to chemical polymers due to their eco-friendly and biodegradable characteristics. Lignin-based polymers include phenol-formaldehyde adhesives (168), phenolic resins (169), and polyurethane foams (170). Similarly, lignosulfonates are broadly applied for sequestration of heavy metals, binders, and emulsifiers (166).

The high aromatic content and amorphous character of lignin make it particularly suited for the production of graphene structures such as carbon fibers. However, this process demands a high purity of lignin as well as a narrow molecular weight distribution (171). Lignin-based hydrogels, polymer-based networks that swell when in contact with water or aqueous solvents, have been prepared (172) using graft copolymerization or chemical cross-linking. Evaluation of a lignin-grafted copolymer, specifically, lignin-*graft*-poly(methyl methacrylate-*co*-butyl acrylate), revealed improved thermoplastic elastomeric properties (173) compared to polymer without the lignin and an elastic strain recovery of close to 70%.

Nanotubes synthesized from lignin using alumina membrane templates have very promising features, such as ease of functionalization, auto-fluorescence that allows easy detection, and flexibility. Based on cytotoxicity studies, human cells have a high tolerance for lignin-based nanotubes, which made them highly efficient vehicles for gene delivery into human cells (174).

When lignin nanocrystals were confined on reduced graphene oxides as an electrochemical energy storage material, they showed excellent capacitive characteristics, demonstrating the potential of lignin for development of advanced renewable electrodes for energy storage (175).

Vanillin is a high-value compound of food, beverages, pharmaceuticals, and fragrances. While the initial process of obtaining this from lignosulfonates created a significant environmental burden, a new process has been developed by Borregaard in Norway that is more eco-friendly (176). More recently, a procedure for obtaining vanillin from Kraft lignin has been developed (87).

CONCLUSIONS

Fungi promise to provide a valuable source of lignin-acting enzymes for deconstruction and conversion. As the technologies developed to liberate sugars from biomass for subsequent fermentation into specialty biofuels and bioproducts become increasingly efficient, more attention is being given to the lignin fraction as a source for high-value products. Valorizing lignin as more than a burning fuel to generate heat will not only further develop a renewable biorefinery, but will also add significantly to the economic sustainability of second-generation biofuel plants. However, to achieve this, pretreatments that leave a convertible form of lignin will need to be further refined. Even more importantly, biological, thermochemical, and hybrid processes must continue to be developed to convert lignin streams into useful products. These processes will have to enable economically viable conversion of the carbon in lignin to specialty fuels and chemicals. However, it should not be forgotten that fungal lignin-modifying enzymes and chemistries have developed over millions of years to deal with the native lignin present in plant biomass. Emerging fungal genomes, and characterization of both endogenous and exogenous enzymes, hold great promise for utilizing lignin.

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Fungi as a Source of Food

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INTRODUCTION

Since ancient times, humans have used fungi as food sources (1, 2). The edible sexual structures of basidiomycetes and ascomycetes (e.g., truffles), the so-called mushrooms, are produced mostly in wood because many fungi are tree symbionts or decayers of tree tissues. These fruiting bodies represent a rich source of proteins, with low fat content and otherwise nutritionally quite poor. In some soils, they accumulate pollution (heavy metals and radioactivity) and should only be eaten in moderate quantities. Some mushroom species are considered delicacies (e.g., truffles, boletus, morels), but cultivation attempts have been unsuccessful, with a few exceptions (e.g., *Morchella rufobrunnea*). Only a few saprobic species can be industrially produced, such as *Agaricus bisporus* (3), *Lentinus edodes* (shiitake), and *Pleurotus ostreatus*, with production having mainly taken place in Asia for thousands of years (4). Some other fungi, while not really cultured, are inoculated on trees grown in appropriate natural habitats to increase the production of fruiting bodies, such as for shiitake and oyster mushrooms, with, however, sometimes unpredictable success, as is the case for truffles (5).

A few plant-pathogenic fungi are also eaten, for example, the corn smut fungus, *Ustilago maydis*, which produces black tumors on maize. These tumors are considered a delicacy in Mexico, where it is called *huitlacoche* (6). These fungi can have great cultural significance (7), as do hallucinogenic mushrooms in many traditional cultures (8).

Fungi are also used for the production of fermented food and beverages in all traditional and indigenous cultures in the world. Examples include cheeses, bread, beer, wine, cider, rice, and soy sauce. Humans have exploited the natural abilities of fungi to ferment fruits and grains to produce alcoholic beverages and bread since as early as 6000 BCE (9–11) and for cheese since at least 7500 BCE (12). Yeasts are used for the fermentation of bread, wine, and beer, while filamentous fungi are used for the maturation of cheeses and soy sauce (from soybeans using *Aspergillus oryzae*) (13) and the production of alcohol from rice (yielding sake, also using *A. oryzae*) (14). In the south of France and other regions, a plant pathogenic fungus, *Botrytis cinerea*, is used to concentrate sugar in grape berries before collection, yielding "noble rot," which is a sweet and expensive wine (15). Some other filamentous fungi and yeasts are used to ferment and preserve meat from food spoilage, in particular *Penicillium nalgiovense*, which forms the white crust on salamis (16).

Before Louis Pasteur's research, microorganisms and their importance were not recognized. Humans thus unintentionally used the ability of fungi to ferment and produce alcohol, which inadvertently led to artificial selection of the most effective fungal strains, thus modifying species such as beer yeasts and cheese fungi (17–19). Because these species have acquired new adaptive traits compared to their wild ancestors (17–24), they can be considered domesticated species. They thus constitute excellent models for studying rapid and recent adaptation (18).

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FUNGI AND CHEESE: DOMESTICATION OF *PENICILLIUM* FUNGI

Cheese making by early Neolithic farmers was a major advance in food processing, allowing milk to be preserved in a nonperishable, transportable form and making it more digestible for adults, because cheese contains much less lactose than fresh milk does (25). The earliest evidences of cheese making date from the 6th millennium BCE in Poland, with findings of the presence of milk fat in sieve vessels (12), and from the early Bronze Age (ca. 3,800 years ago), with the discovery of residues of old cheese in tombs (26). The earliest cheeses were simple, made by adding lactic acid bacteria (LAB) to fresh milk to provoke milk curdling and then draining in sieve vessels. The use of different mechanical processes to drain the curd, i.e., carving, brewing, pressing, grinding, heating, and the maturing of these cheeses, appeared later and gave rise to the large variety of cheeses known today (soft cheeses, blue-veined cheeses, hard cheeses, uncooked firm cheeses, and cooked firm cheeses). Microorganisms play a major role in the cheese-making process, from the initial milk curdling by LAB to the maturation step by fungi (including yeasts and molds). Indeed, during ripening, microbiological and biochemical changes directly influence the development of the texture and flavor that make each kind of cheese unique. Primary biochemical changes include (i) lipolysis, in which LAB are the main actors by metabolizing lactose into lactate; (ii) proteolysis, which has a direct influence on flavor through the production of short peptides and amino acids, originating from six primary sources (e.g., the coagulant, the milk, starter LAB, nonstarter LAB), secondary starters (molds such as *Penicillium roqueforti* in blue cheeses), and Gram-positive bacterial microflora on the surface of smear cheeses (27); and (iii) metabolism of residual lactose, lactate, and citrate. Secondary biochemical changes include metabolism of fatty acids and of amino acids by molds such as *P. roqueforti* in blue cheeses and *Penicillium camemberti* or *Geotrichum candidum* in soft cheeses such as Camembert and Brie. Microbial interactions within a cheese are thus crucial to produce a cheese with the desired texture and flavor, making the cheese substrate a complex but very interesting ecosystem in which to study microbial community interactions (28, 29).

Besides the emblematic cheese-making species *P. camemberti* and *P. roqueforti*, cheese producers widely use other important filamentous Ascomycota within the classes of Eurotiomycetes and Sordariomycetes (30). *Penicillium* species (Eurotiales) are used for the production of blue cheeses such as Roquefort and Fourme d'Ambert (*P. roqueforti*) and soft cheeses such as Camembert and Brie (*P. camemberti*). *Sporendonema casei* (Onygenales) is used for the production of firm cheeses such as cantal and Salers. Some *Scopulariopsis* species (*Scopulariopsis candida*, *Scopulariopsis flava*, and *Scopulariopsis fusca*, Microascales) are found in uncooked hard cheeses, including Tomme des Pyrénées and Ossau-Iraty, while *Fusarium domesticum* (Hypocreales) is inoculated for the production of Saint Nectaire and Reblochon. Along with Ascomycota, several Mucoromycotina species from the genus *Mucor*, including *Mucor racemosus*, *Mucor circinelloides*, and *Mucor lanceolatus*, are used for the production of uncooked hard cheeses such as Saint Nectaire and Tomme de Savoie (31). From an ecological point of view, some species are specific to the cheese environment (e.g., *F. domesticum*, *P. camemberti*, *S. casei*, *S. flava*, *M. lanceolatus*) while others are ubiquitous (e.g., *P. roqueforti*, *S. fusca*, *S. candida*). This leads to the questions of whether and how these species have adapted to this particular man-made, nutrient-rich ecological niche.

P. camemberti and *P. roqueforti* are ideal models for studying parallel adaptation because they are distantly related and have completely different domestication histories (32). *P. camemberti* is a human-created species, being a clonal lineage selected in the late 19th century for its white color and its fluffy, downy texture. It is thought to be derived from the blue-gray mold *Penicillium commune*, a species complex now split into *Penicillium biforme* and *Penicillium fuscoglaucum* (32, 33). *P. camemberti* is found only in the cheese environment and is inoculated in soft cheeses such as Camembert and Brie; no genetic or morphological diversity has been found in this species so far. In contrast, *P. roqueforti* is a common food spoilage agent in refrigerated stored foods, meat, and wheat products because of its ability to tolerate cold temperatures, low oxygen concentrations, alkali, and weak acid preservatives; it also occurs in silage and even in forest soil and wood (34, 35).

P. roqueforti has occurred in blue cheeses since at least antiquity (36–38). The fungus was originally not inoculated during blue cheese production but appeared spontaneously. At the beginning of the 19th century, environmental spores of *P. roqueforti* were collected from rotten bread left on purpose to rot on shelves in caves. Later, breads were inoculated with *P. roqueforti* spore powder kept from previous inocula, and strains that yielded good cheese were selected. For the past 30 to 40 years, the inoculated strains have been much more carefully controlled. Inoculated spores now mostly come from sterile *in vitro* conditions and derive from monospore isolations. Such controlled cultivation aims

to avoid sanitary issues and to render the cheese maturation process more repeatable and reliable (36–38). In contrast to *P. camemberti*, *P. roqueforti* shows substantial within-species genetic and phenotypic diversity, as well as genetic subdivision (39, 40). In particular, different genetic clusters of *P. roqueforti* seem to have been independently domesticated (41), and subclusters correspond exclusively to particular designation of origins, notably the Roquefort cheeses (41).

Recent studies compared the genomes of the two emblematic cheese species *P. camemberti* and *P. roqueforti* to eight other *Penicillium* species, four of which were isolated from the cheese environment but considered as contaminants by producers (*Penicillium paneum*, *P. fuscoglaucum*, *P. biforme*, and *P. nalgiovense*) (17, 19). The study also included a *Penicillium* species isolated as a contaminant in the meat environment (*Penicillium carneum*), the penicillin-producer *Penicillium rubens* and its close relative *Penicillium chrysogenum*, and the necrotrophic pathogen *Penicillium digitatum*, which is specialized to citrus. The genomic comparison revealed multiple horizontal gene transfers (HGTs) likely involved in cheese adaptation (17, 19). HGT is the process whereby genetic material is transferred between species by means other than vertical descent. In particular, two genomic regions of several hundreds of kilobases, named *Wallaby* (17) and *CheesyTer* (19), were shown to share a nucleotide identity of almost 100% among all cheese *Penicillium* species while being absent from noncheese species. These horizontally transferred regions (HTRs) were unique when compared to other HTR cases cited in the literature by (i) their size (*Wallaby* is ca. 600 kb long, encompassing 2% of the genome of *P. roqueforti*, and *CheesyTer* is 80 kb long), (ii) their eukaryotic origin (likely among cheese-associated *Penicillium* species), (iii) the number of species in which the same regions have been horizontally transferred, and (iv) the very recent date of the transfers, likely associated with the human history of cheese production (17, 19, 32, 42). These HTRs are flanked by copies of a particular family of transposable elements, namely, the *i* non-long terminal repeat (LTR) retrotransposons, that are always found clustered around putative HTRs in *P. roqueforti*. In contrast, the other transposable element families such as *copia* or *gypsy* LTR retrotransposons are widespread along the *P. roqueforti* genome (19). This supports the idea that *i* non-LTR retrotransposons may be involved in the horizontal transfer of these regions.

The gene content of these HTRs suggests that *Wallaby* and *CheesyTer* may play a role in the cheese adaptation. Indeed, two genes in *Wallaby*, named *paf* and *Hce2*, encode proteins previously described to be involved in interactions with other microorganisms (17). Genes coding for a lactose permease and a beta-galactosidase were found in *CheesyTer*, suggesting a role in the metabolism of the cheese substrate (19). These genes were found to be overexpressed during the first days of cheese maturation in an industrial Canadian Camembert-type cheese (19, 43). Experiments of growth and competition on different media using *P. roqueforti* strains have further supported a role of these HTRs in adaptation to cheese. Indeed, *P. roqueforti* was found to display polymorphism regarding the presence of *Wallaby* and *CheesyTer*: strains isolated from other environments than cheese did not carry either of the two HTRs, and among cheese strains, only a subset of strains carried the HTRs, and always either both *Wallaby* and *CheesyTer* or neither of them. Strains carrying the two HTRs grew significantly faster on cheese medium and significantly more slowly on minimal medium (19). Furthermore, strains carrying the HTRs showed a significant competitive advantage, both against *P. roqueforti* strains lacking the HTRs and against other *Penicillium* species also lacking the HTRs (19).

In conclusion, *Penicillium* fungi show footprints of rapid adaptation through frequent HGTs that have occurred under selection in the human-made cheese environments. This shows the importance of HGTs, in particular in the food environments. Now that we know about the ability of microorganisms to exchange genes through nonvertical descent and even between distant species, the use of genetically modified organisms in the food chain should be avoided.

Finally, domestication of cheese fungi has impacted their mode of reproduction. Indeed, industrial companies replicate their strains exclusively clonally, without sex. This together with bottlenecks and strong selection may contribute to the degeneration of cheese strains in the form of reduced fertility as occurs in domesticated plant and animal species (44–47). Investigations of premating (i.e., prevention of syngamy between individuals) and postmating fertility (i.e., syngamy occurs but the progeny is not viable or fertile) between and within genetic clusters of *P. roqueforti* have suggested reduced fertility of cheese strains (48). This degeneration has occurred independently in two cheese lineages, representing convergent evolution on a very short timescale.

YEASTS IN THE WINERY, BAKERY, AND BREWERY

Humans have used *Saccharomyces* yeasts for thousands of years for brewing, baking, and wine making.

Because yeasts are able to convert sugars into alcohol, they were used primarily as effective ways to preserve the quality and safety of foods and beverages, because high concentrations of ethanol are toxic for most other microbes. During food fermentation, yeasts convert sugars to ethanol and carbon dioxide under both anaerobic (fermentation) and aerobic conditions (known as the Crabtree effect) (49, 50).

Saccharomyces species are ascomycetous yeasts (51) that are model systems for ecology, genetics, and evolution (52), particularly at the genomic level (53). Studies have focused primarily on domesticated forms: besides the famous *Saccharomyces cerevisiae*, other closely related species belonging to the *Saccharomyces sensu stricto* complex and several important interspecific hybrids have been isolated from fermented food (bread) and beverages (beer, wine, cider, and sake). Their relationships are shown in Fig. 1. The abundance and variety of hybrids in fermented products are intriguing (54) and suggest that such hybrids could have selective advantages over parental species (55). It is possible that stressful conditions in fermentation processes promote hybridization events (52). The persistence of these hybrids in the long term has been questioned (56), but there is some evidence that they undergo progressive genome stabilization (54). Comparative genomic studies of hybrids and ideally of the progenitors of the hybrids are essential to unravel the process of domestication and the impact of domestication on genome architecture and function (57). The discovery of progenitors is sometimes challenging, as it was for the lager beer hybrid *Saccharomyces pastorianus*. This organism has been known to be an interspecific hybrid for more than 3 decades (see below for more details), but the second progenitor was only recently identified as *Saccharomyces eubayanus* (58). The fact that new food-associated *Saccharomyces* species are still being discovered points to our still-limited knowledge about the diversity of yeasts.

The origin and the natural environment of the baker's yeast *S. cerevisiae* have long remained a mystery. Given their ability to ferment sugars, *Saccharomyces* yeasts have been sought in mature and rotted fruits, in sugar-rich plant exudates, and in nearby soils (59). It seems that oak trees in the Northern Hemisphere (59–63) and *Nothofagus* trees in the Southern Hemisphere (58) could be important in the natural life cycle of *Saccharomyces* yeasts. Currently, *S. cerevisiae* seems to have a global distribution in wild and human-associated environments (56). In contrast, other species such as *Saccharomyces paradoxus* and *Saccharomyces kudriavzevii* seem to be more limited in their

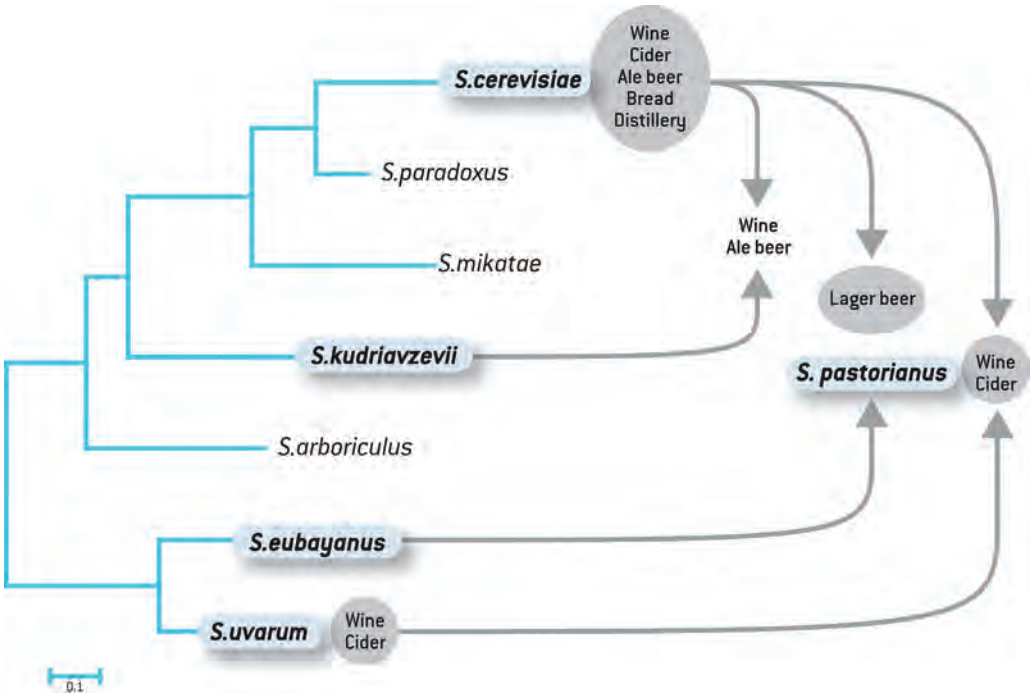


Figure 1 Relationships between *Saccharomyces* species and their industrial hybrids. Tree topology was obtained using a subset of 25,000 single nucleotide polymorphisms selected after genome alignment.

geographical distribution, with strong spatial genetic subdivision between populations suggesting limited dispersal (56). Recently, extensive field surveys conducted in China revealed substantial and novel genetic diversity among wild isolates, suggesting East Asia as a possible center of origin of *Saccharomyces* yeasts (59, 63).

The life cycle of *Saccharomyces* yeasts is well documented in the laboratory (52), but little is known about their life cycle in natural populations. This is true even for the well-studied *S. cerevisiae*; how strains disperse and how and where they reproduce are unknown (64). Recent data have confirmed the importance of insects in their dispersal, e.g., fruit flies for *Candida* species, which are members of the same family (65). Social wasps host *S. cerevisiae* in their intestine and disperse them in the wild (66). More importantly, the wasp intestine provides environmental conditions favoring mating and outbreeding of *Saccharomyces* yeasts (67), an important finding that may explain the high levels of variability and mosaicism observed in *S. cerevisiae* populations. Genotyping and population genomic studies have indeed revealed a complex population structure of *S. cerevisiae* (68–72), with, on the one hand, well-delimited lineages of particular geographical or technological origins (wine, sake, beer) and, on the other hand, mosaic lineages, with recombinant genomes likely originating from admixture after human-associated dispersal. The mosaic lineages include clinical isolates (73), wine strains (74), bakery isolates, and some laboratory strains (64, 70). Strains also cluster according to their ploidy level and type of mating compatibility (75).

Altogether, these results suggest different domestication histories for beer, bread, and wine strains. The following sections focus on recent progress made in understanding the origin and the impact of domestication of *Saccharomyces* yeasts in different fermentation usages.

Wine Fermentation

The first evidence of wine production by humans is dated to the Neolithic period. The presence of tartaric acid and terebinth resin was detected in a pottery jar dated to 5400 to 5000 BCE in Hajji Firuz, Iran (10). Evidence was also provided that the yeast *S. cerevisiae* was responsible for wine fermentation in Egypt by at least 3150 BCE (76). Wine fermentation technologies expanded from Mesopotamia toward Europe and subsequently spread to the New World (77).

Wine alcoholic fermentation is a complex process involving many yeast genera and species that are part of the grape berries' microflora (78). The first stage of wine fermentation involves mainly non-*Saccharomyces* yeasts. However, due to its outstanding fermentative abilities in anaerobic conditions and to its high tolerance to ethanol, *S. cerevisiae* rapidly dominates alcoholic fermentation and is responsible for the degradation of the majority of sugars in alcohol. In the 1970s, the practice of inoculation with selected pure cultures of *S. cerevisiae* in the form of active dry yeast became widespread, and this has enabled better control and reliability of the fermentation process, contributing to the improvement of wine quality. In addition to *S. cerevisiae*, other *Saccharomyces* species and a growing number of interspecies hybrids involved in wine fermentation have been recently identified and characterized.

The fermentation of grape juice exposes yeast to a variety of stresses, including high osmolarity reflecting high sugar concentrations (180 to 260 g/liter), low pH (3 to 3.5), the presence of added sulfites (40 to 80 mg/liter), anaerobiosis, limiting amounts in nutrient (nitrogen, lipids, and vitamins), and ethanol toxicity. During this process, the majority of hexoses are converted into ethanol and CO_2, and a small fraction of sugars is used to synthesize the anabolic precursors required for biomass formation. Various by-products, including glycerol, carboxylic acids, aldehydes, higher alcohols, esters, carbonyl compounds, and sulfur compounds, are also formed, deriving from the degradation of sugars, amino acids, and fatty acids. Moreover, yeast can also convert nonaromatic grape precursors into aromas (monoterpenes and thiols). These metabolites play a crucial role in the organoleptic balance of wines (79).

Wine fermentation comprises mainly a lag phase and a short growth phase followed by a stationary phase, during which most of the sugar (between 50 and 80%) is fermented. Nitrogen is the main limiting nutrient responsible for cell proliferation arrest, although the availability of other micronutrients such as lipids and vitamins can also be a limiting factor (80). Nutrient imbalance affects yeast fermentation capacity, resulting in stuck or sluggish fermentations, and can also affect the production of volatile compounds and the organoleptic balance of wine.

Today, more than 200 wine strains of *S. cerevisiae* are used to inoculate grape musts. These strains have been selected from spontaneous fermentations or selected in the vineyard environments according to particular fermentative capacities, tolerance to stresses, particularly ethanol and sulfites, and the limited production of undesirable compounds such as H_2S and acetate.

Diversity of wine yeasts and evidence of adaptive evolution to the wine environment

In the past decade, various studies based on multigene sequencing (59, 66, 68), microsatellite analyses (69),

tiling array hybridization (81), low-coverage whole-genome sequencing (70), or restriction-site-associated sequencing (Rad-seq) (82) have provided deep insights into the population structure and evolutionary history of *S. cerevisiae*. Genome analyses of *S. cerevisiae* isolates from different sources have led to the identification of five distinct lineages based on their technological and geographic origin (West African, Malaysian, North American, sake, and European/wine). Strains with mosaic genomes resulting from crosses between these lineages have also been identified (70, 81). These studies and earlier findings (68–70, 81–83) have shown that wine yeasts belong to a distinct phylogenetic group with low diversity and have suggested an initial domestication event followed by an expansion of the population favored by human activity. A recent study increased the number of collections of natural lineages, especially oak-associated wild isolates, and used population genomics analyses to analyze a balanced number of anthropic and natural *S. cerevisiae* strains (84). This study identified a new Mediterranean oak population that seems to be the closest wild relative to the wine lineage known to date. The estimated divergence time between the two populations is consistent with the first historical evidences of wine making. Furthermore, three horizontally acquired regions largely widespread in wine yeasts and containing genes relevant to wine making (24) are absent in the wild Mediterranean oak group. These results suggest that the common ancestor of these two populations provided the wild genetic stock that underwent domestication and gave rise to wine yeasts (84, 85).

An increasing number of studies in the past decade have focused on the understanding of the genetic and functional underpinnings of human-driven selection of wine yeasts over millennia. Wine yeast strains demonstrate better resistance to chemical compounds such as copper (86, 87) used in vineyards or sulfites (88, 89) added in grape must. Several wine yeast strains also have the ability to use xylose as a carbon source (90) or different types of di-tripeptides as nitrogen sources (22, 91, 92), and recent data suggest that they might produce more fruity aromas than do strains from other origins (93). Identifying the variations contributing to the properties of wine yeast and deciphering the mechanisms that participate in these evolutionary processes remain major challenges. Several molecular mechanisms may have contributed to the adaptive evolution of wine yeast genomes, including single nucleotide polymorphisms, chromosomal rearrangements, gene copy number variations, introgressions, HGT, and interspecific hybridization (21, 94–96). A hallmark of adaptation has been detected in the genome of so-called flor yeasts. Flor yeasts are closely related to wine strains but have distinct capacities such as the ability to form a biofilm on the surface of wine after fermentation and to develop oxidative metabolism in the presence of a high ethanol concentration and a low amount of fermentable sugar, mainly fructose (97). Flor strains have acquired two mutations in the promoter and the coding regions of the gene *FLO11* encoding a glycosylphosphatidylinositol-anchored cell surface glycoprotein (flocculin) required for pseudohyphal growth and biofilm formation (98, 99), resulting in enhanced cell adhesion. In addition, flor strains possess a fructophylic allele of the *HXT3* hexose transporter previously identified in an industrial strain (100) and a high-affinity fructose transporter, *FSY1*, which may provide an adaptive advantage after alcoholic fermentation when traces of fructose remain in the fermented must but glucose is exhausted (101).

A striking example of yeast adaptation to current wine-making environments is the response of these fungi to chemicals used in the vineyard and winery environments. Elevated copper tolerance in the European wine and sake lineages has been associated with a copy number variation of *CUP1*, which encodes a copper-binding metallothionein (87). The recent resequencing of the genome of 100 yeast strains revealed that some strains of the wine/European cluster could possess up to 18 copies of the *CUP1* gene (102). The acquisition of this trait can be associated with the use of copper sulfate in vineyards, which has been used as a fungicide against powdery mildew since the 1880s (86).

Genomic variations have also been gained by wine yeasts as a response to the use of sulfite. Two reciprocal translocations, between chromosomes VIII and XVI or XV and XVI, have been detected among wine yeasts. These translocations result in the overexpression of *SSU1*, encoding a sulfite pump and thus conferring a high level of sulfite resistance (89, 103, 104).

Several signatures of introgressions with potential adaptive roles have been identified in wine yeasts. A large *S. paradoxus* introgressed region, identified in commercial *S. cerevisiae* wine yeast strains (105), contains the *S. paradoxus SUC2* gene, which encodes sucrose-hydrolyzing invertase, and a gene similar to *S. cerevisiae HPF1*, which encodes a glucan alpha-1,4-glucosidase that, when overexpressed, reduces protein haze formation in white wines (106). The cryotolerant species *Saccharomyces uvarum* is also found in wine fermentation. A recent study of the population structure and diversity of this species revealed multiple introgressions from other *Saccharomyces* species, especially

from *S. eubayanus* (107). These introgressed regions are enriched in functions involving nitrogen metabolism, suggesting that these regions might confer an advantage under nitrogen-limiting wine fermentation conditions.

Horizontal gene transfer also significantly contributes to the adaptation of wine yeasts. Three large genomic regions, called A, B, and C, acquired through independent HGT events from distant yeast species were identified in the genome of the commercial *S. cerevisiae* wine yeast EC1118 (24) and were furthermore shown to be widespread among wine yeasts and some mosaic genomes (24, 71, 108). These genomic islands contain 39 genes encoding potentially important metabolic functions in wine making (sugar and nitrogen metabolism), suggesting a role in adaptation to the wine environment. The yeasts *Zygosaccharomyces bailii* and *Torulaspora microellipsoides* were identified as the donors of regions B and C, respectively (22, 24). For region C, the transfer was dated to approximately 2,000 years ago (22). Evidence was provided of an initial insertion of a 158-kb genomic fragment from *T. microellipsoides* into the *S. cerevisiae* genome, followed by several rearrangements including gene losses and gene conversion between two tandemly duplicated *FOT* genes encoding oligopeptide transporters (22). Interestingly, *FOT* genes are strongly conserved in wine yeasts. These genes confer a strong competitive advantage during grape must fermentation by increasing the amount and diversity of oligopeptides that yeast can utilize as a source of nitrogen, thereby improving biomass formation, fermentation efficiency, and cell viability (22).

Thus, the acquisition of *FOT* genes has favored yeast adaptation to the nitrogen-limited wine fermentation environment. Furthermore, *FOT*-mediated uptake of peptides rich in glutamate substantially affects central carbon and nitrogen metabolism, resulting in decreased acetic acid production and increased ester formation, which might improve the organoleptic balance of wines (109).

Wine hybrids: characterization and advantages

An increasing number of interspecific hybrids have been identified in the wine environment, including *S. cerevisiae/S. kudriavzevii* (110–117), *S. cerevisiae/S. uvarum* (113, 118–120), and *S. cerevisiae/S. kudriavzevii/S. uvarum* (23, 110). These hybrids present several advantages in wine fermentation such as increased tolerance to various stresses and robustness compared with the parents (54, 114, 115, 121–123). *S. kudriavzevii* and *S. uvarum* are better adapted to growth at low temperatures compared to *S. cerevisiae*, whereas *S. cerevisiae* is more alcohol- tolerant. The natural hybrids between these species have adapted to growth under ethanol and temperature stress through the inheritance of competitive traits from one or another parental species (124). These hybrids have potential value under conditions of fermentation of white wines at low temperatures (10 to 15°C) that minimize the loss of aromatic volatile compounds. Hybrids of *S. cerevisiae* × *S. kudriavzevii* produce more esters, fusel (>2 carbon) alcohols (112, 115, 121), and thiols derived from non-aromatic precursors present in grapes than *S. cerevisiae* does (125, 126). The abundance of these hybrids could reflect an adaptive advantage, but it is also possible that stressful conditions trigger hybridization events (52).

Improvement of wine yeast

Although many benefits to wine production have been gained through the selection of strains with suitable properties, efforts can still be deployed to improve wine yeasts for various traits, e.g., improvement of stress tolerance, fermentative performance, aroma properties, or low ethanol production. Non-genetically modified organism strategies based on rational genetic strategies to exploit existing natural diversity have proven successful in the past decade to develop strains with improved traits. These strategies build on the identification of desired traits in the yeast genome by quantitative trait locus analyses. Quantitative trait loci controlling the formation of acetic acid, aroma compounds, SO_2 production, nitrogen utilization, or ethanol resistance have been identified (127). Once quantitative trait loci have been identified, the desired alleles can be transferred into a new strain by breeding using marker-assisted allele transfer. In addition, several strains with improved phenotypes have been developed using adaptive laboratory evolution approaches, which involve the propagation of a microbial population under defined selective conditions for prolonged periods of time and the selection of better-fitted mutants. These strategies have allowed the development of wine yeast strains overproducing esters, which are major determinants of the fruity character of wines (128) and strains with reduced ethanol yield (129, 130). The construction of interspecific hybrids to generate new combinations of genes conferring new properties has also been performed (131, 132).

Baker's Yeasts

S. cerevisiae is also known as baker's yeast. Bread-making scenes have been discovered on reliefs in several tombs dating from ancient Egypt, and the analysis of pottery containing remains of cereals and bread has allowed the investigation of ancient Egyptian baking

methods (11). However, the origin and expansion of the process of leavened bread is poorly documented, and it is unclear whether yeasts came from cereals or from the process of fermenting beer (133). In countries that produce beer, ale yeast was used in bread production, and a portion of the yeasted dough was recycled by blending it with fresh water and flour for formation of the next dough. The production of baker's yeast exclusively for bread dough production has been dated from the late 19th century. Initially, this was done by production of distiller's yeast on mashed grains, and later with the less expensive source of assimilable sugar, molasses (134).

Most of the bread consumed today is leavened with the commercial baker's yeast, *S. cerevisiae*. However, the consumer demand for sourdough bread is increasing, and some bakers use sourdough containing natural lactic bacteria and yeasts as an alternative to commercial yeasts (see reference 135 for a review). The diversity of yeast strains is high in French natural sourdoughs, with *Kazachstania* species dominating and *S. cerevisiae* being an uncommon species (136). This suggests that *S. cerevisiae* does not compete well with other sourdough and yeast species.

Baker's yeast provides the gas required to leaven the dough through the alcoholic fermentation of sugars, in particular maltose, but yeast also influences the bread texture and taste. In contrast to beer and wine, bread dough is a solid fermentation occurring in a reduced level of free water, and the cellular physiology of the yeasts is likely different than in liquid fermentation. The study of the yeast transcriptome throughout the bread dough fermentation process has revealed three phases during which protein- and RNA-coding genes are differentially expressed (137). At the onset of fermentation, expression of glucose-regulated genes changes dramatically, and the osmotic stress response is activated. The middle fermentation phase is characterized by the induction of genes involved in amino acid metabolism. Finally, in the last phase, cells suffer from nutrient depletion and activate pathways associated with starvation and stress responses. Altogether, the results have shown that cells embedded in a solid matrix such as bread dough suffer severe osmotic stress.

Baker's yeasts have lower genetic diversity and a higher number of chromosomes than other *S. cerevisiae* lineages used for wine, sake, and beer fermentations (138). But unlike brewing yeasts, which are allotetraploid, baking yeasts are autotetraploid, displaying four complete chromosome sets arising from the same species. Usually transitory, the autotetraploid status may have occurred very recently and/or may have been maintained by human selection because of metabolic benefits for fermentation (75). The genome analysis of three bread strains has shown the mosaic nature of their genomes, with some regions very similar to those present in wine strains; some others being more related to sake, oak, or palm wine yeast genomes; and some that were unique (70, 138). This suggested that baker's yeasts have arisen from crosses between wild lineages that had appropriate fermentation properties (138).

Improvement of Baker's Yeast

The evolution of bakery technologies has highlighted a need to improve yeasts for their tolerance to various stresses, including high sugar concentration (in sweet dough), drying, and freezing (in relation to the increase of production of dry and frozen dough, respectively), as well as for their performance in the fermentation of dough sugars and for their growth rate and biomass yield relevant to the production of the baker's yeast itself (138, 139). Different genetic engineering strategies have been applied, in particular manipulation of the small osmolyte pathways, including those of glycerol, proline, and trehalose (137, 138). Experimental evolution has been used to enhance the freeze-stress tolerance of industrial baker's yeast cells by generating adaptations to growth at low temperature (140). Two new baker's yeast strains obtained by hybridization between commercial baker's yeasts have shown improved fermentation abilities and cryoresistance (141).

Brewing Yeasts

Beer is the most widely consumed alcoholic beverage in the world, with a total consumption volume of 176.4 billion liters in 2014, corresponding to total revenues of $464.4 billion (142).

Water, hops, malt, and yeasts are the four main ingredients for manufacturing beers (143). Hops give beer its typical bitterness and aroma. Malt is obtained by inducing the germination of grains, mainly barley grains, for a period of time necessary for enzymes to degrade complex molecules, such as starch and proteins, into forms that can be assimilated by yeasts. To stop the process, water is removed by fresh air or heating, to obtain green to pale or dark dried malts, respectively. This manipulation of the process impacts the beer's characteristics, in particular the color of pale to dark beers. Barley or malt is roasted to make the typical dark stout beers.

Generally, the yeasts used for beer production belong to the *Saccharomyces* genus. Depending on their physiological characteristics, and on the quality of other ingredients, two main types of beers are produced, i.e.,

ale or lager-style beers. Ales are brewed at a relatively high temperature (15 to 26°C) with top-fermenting *S. cerevisiae* strains, which rise to the surface of the fermenting wort after cell aggregation during the flocculation process. Lager beers are brewed at lower temperature (5 to 14°C) with bottom-fermenting yeasts that have a tendency to sediment after flocculation and sink to the bottom of the fermenters. Ales have been brewed since ancient times, possibly as early as 6000 BCE (144). In contrast, lager beer production was limited to cool seasons but expanded with the invention of the refrigerating machine by Linde in 1871, allowing the necessary cool temperatures for bottom fermentations to be achieved during all seasons. Another major advance was the development of pure cultures of yeasts. Previously, brewmasters selected best batches and yeasts were reutilized from batch to batch. At the time, problems in the end product such as bitter taste and bad odor frequently occurred. In 1883 E. C. Hansen, who worked at the Carlsberg Laboratory in Copenhagen, developed an effective technique to obtain pure cultures of yeasts. This technique was essential to standardize beer production by selection of desirable yeasts and to the standardization of yeast cultures all over the world (145). Today lager beer production dominates the market.

A fascinating and ongoing research story began when the lager yeast, whose current name is *S. pastorianus* (syn. *Saccharomyces carlsbergensis*) (146), was discovered to be an interspecific hybrid showing high similarity in nucleotide sequence to both *S. cerevisiae* and *Saccharomyces bayanus* (147). A microsatellite analysis suggested that the ancestral *S. cerevisiae* parent of the hybrid came from the ale-brewing process, because ale and lager beer yeasts clustered together and were distinct from bread and wine yeasts (69). This result was confirmed by sequence data (148, 149). The second parental species was suggested to be closely related to *S. bayanus* (23, 150, 151) until the discovery of a better candidate, the cryophilic species *S. eubayanus*, first isolated in Patagonia (58) and recently also in China (63). *S. pastorianus* strains have greater mitochondrial DNA sequence similarity to the Tibetan strain BaiFY1 than to the type strain CBS12357 isolated in Patagonia (152), in agreement with previous genomic data (63). These data suggest a common origin of the domesticated *S. pastorianus* strains with the wild Tibetan strains and support a scenario of continental migration of *S. eubayanus* from China to Europe along the Silk Road (153), rather than by overseas transport from Patagonia to Europe as originally suggested (58). It is thought that the hybridization event probably occurred quite recently, about 500 years ago (153). To elucidate the date and circumstances of the process, more isolates from more diverse origins in the world and particularly along the hypothesized migration routes are needed for population genomics studies.

The genetic diversity in *S. pastorianus* populations is much lower than that in ale yeasts (154). Two groups were identified in *S. pastorianus*, called Saaz (or group I) and Frohberg (group II), after the locations in Bohemia and Germany in which original strains were derived (145). Each group has specific brewing abilities and produces different flavors. Saaz strains have a greater cold tolerance than Frohberg strains but relatively poor fermentation performance due to their inability to utilize maltotriose (155, 156). This deficiency may have contributed to favor the use of Frohberg strains in modern brewing companies (155). Not surprisingly, genetic differences were found between the two groups, including genome rearrangements, copy number variations, ploidy differences, mitochondrial and nuclear DNA sequence polymorphisms (148, 152), and transposon distribution (157). More precisely, the genomic content inherited from hybridizing ancestral species appeared different in the two *S. pastorianus* groups. Saaz strains are triploid, containing less than one haploid genome of *S. cerevisiae* and more than a diploid *S. eubayanus* genome (156), while Frohberg strains are tetraploid, containing one full diploid *S. cerevisiae* genome and one full diploid *S. eubayanus* genome (149, 152, 156, 158).

To date, it is not known whether the Saaz and Frohberg lineages arose from single or separate hybridization events. The differences between the two groups suggest separate hybridization events, in particular the high level of variation in telomeric sequences and LTR-transposon copy number and location (157), the difference in ploidy (148) and in single-nucleotide variants (152), the different rates of evolution in the two subgenomes since hybridization (159), and the pattern of presence/absence variation of the lager-specific genes that are located in the subtelomeric regions (160). Baker et al. (159) suggested that hybridizations may have involved nearly identical *S. eubayanus* parents but more diverse *S. cerevisiae* parental strains. Two classes of *S. cerevisiae* parental strains were identified as a Foster-O-like ale yeast and a stout yeast, respectively, for the groups I (Saaz) and II (Frohberg) hybrid lineages (160). The main argument favoring a single hybridization event at the origin of the two groups is that the two lineages share at least three chromosomal translocations between *S. cerevisiae* and *S. eubayanus*

genomes, with identical breakpoints at the nucleotide level (152, 156).

Okuno et al. (152) proposed two scenarios with at least one common hybridization event to reconcile all the data (Fig. 2). Scenario A proposes hybridization between diploid parental strains as suggested in references 144 and 156 and chromosomal deletion to explain the massive reduction of the *S. cerevisiae* genome in the Saaz type. Scenario B involves a hybridization event between haploid *S. cerevisiae* and diploid *S. eubayanus* leading to a triploid hybrid ancestral Saaz group. To explain the tetraploid Frohberg type, an additional hybridization between the F_1 hybrid and another *S. cerevisiae* strain was suggested, as proposed by Baker et al. (159) and Monerawela et al. (160). The instability in the nascent allopolyploid hybrid genome may have contributed to rapid evolution of the yeast to tolerate the conditions prevalent in the brewing environment (153), particularly the fermentation temperatures, and to the selection of new biochemical activities (85). As a result, amplification and deletion of genes and/or pseudogenization would have occurred at rearrangement breakpoints on different chromosomes in lager hybrids. An increase in the number of copies of genes involved in maltose utilization has been observed on chromosome II. Several breakpoints have led to changes in flocculation genes, particularly the elimination of the *S. cerevisiae* copy of the *FLO5* gene, but the apparent retention of the *S. eubayanus* version of the gene known as Lg-*FLO1* (148, 161). The Frohberg genome was shown to carry inactive copies of both *S. cerevisiae* and *S. eubayanus SUL1* sulfate transporters while retaining functional versions of the two *SUL2* genes, which are more efficient under fermentation conditions (58), sulfite formation being important in lager brewing because it is an antioxidant and flavor stabilizer (139).

The impact of domestication has also been investigated in terms of selective pressure that may have occurred on genes, and particularly on metabolic genes that are important for brewing (159). A global increase

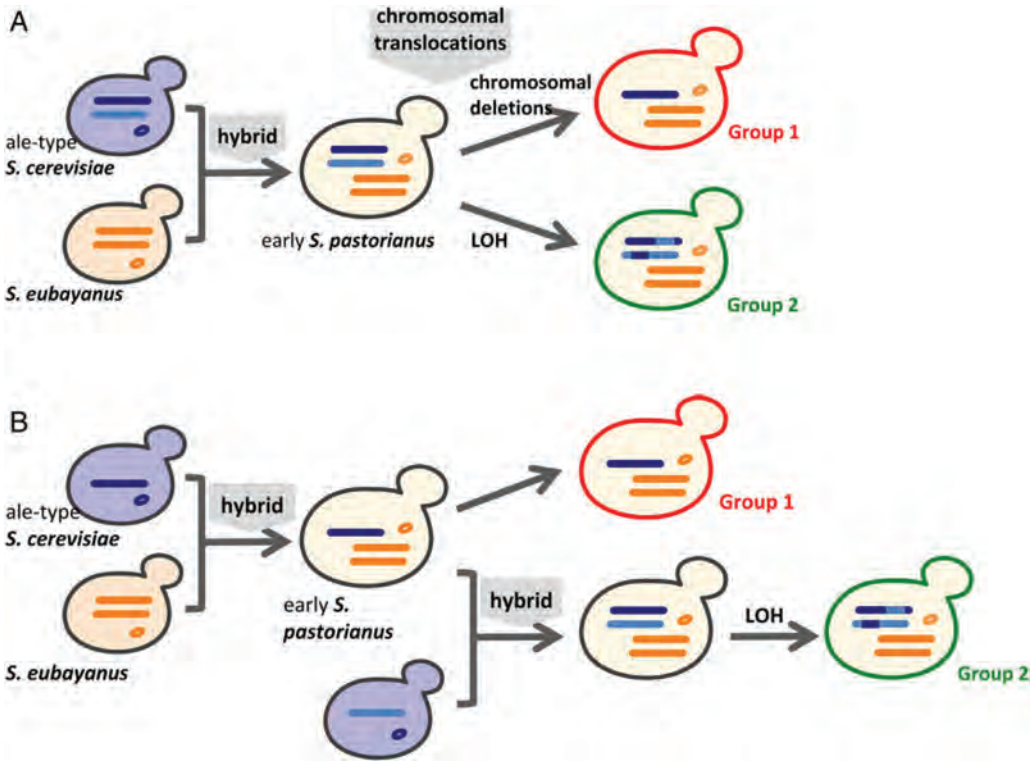


Figure 2 Two hypotheses regarding origins of *S. pastorianus* based on shared chromosomal translocations and differences in ploidy between groups 1 and 2. (**A**) Hybridization between diploid Sc and Se types occurred before chromosomal translocations, whereas chromosomal deletions occurred only in ancestral group 1 strains. (**B**) After hybridization between haploid Sc and diploid Se types and chromosomal translocations, ancestral group 2 strains gained another Sc type (i.e., a second hybridization event occurred). Chromosomal deletion or loss of heterozygosity (LOH) explains single nucleotide polymorphisms observed when comparing reference genomes.

in the fixation of nonsynonymous substitutions in the hybrid *S. eubayanus* subgenome of *S. pastorianus* in comparison to the genome of a wild strain of the parental *S. eubayanus* species was observed and was interpreted as a relaxation of purifying selection during domestication (159). Some genes showed footprints of positive selection, with particularly high rates of protein sequence evolution in both Saaz and Frohberg lineages. However, different nonsynonymous changes have been acquired in the two lineages, indicating convergent adaptations to the same environment (159). Examples include genes encoding global transcription factors or factors required for the expression of enzymes specialized for ethanol oxidation rather than fermentation, as well as genes involved in the regulation of other genes related to the utilization of ethanol (159).

Novel lager brewing yeasts

The recent discovery of *S. eubayanus* opened up the possibility of using this species alone as a lager brewing strain (153, 155) and to create novel artificial hybrids (162, 163). The best of these novel hybrids have shown growth at a broader range of temperatures, a high fermentation capacity in laboratory-scale lager beer fermentations, and desirable aromatic profiles that were significantly different from the profiles produced by the currently exploited lager yeasts. Hybrids previously obtained by breeding lager yeasts with *S. cerevisiae* ale strains have shown improved stress resistance, as seen in the ale parent, including improved survival at the end of fermentation (164).

A. oryzae in Japanese fermentations

A. oryzae has been used for hundreds of years in Japanese fermentations to produce traditional alcoholic drinks, sauces, and condiments. It is one of the most potent secretory producers of proteins among filamentous fungi, and its use is now extended to the commercial production of enzymes in modern biotechnology (165, 166). In Japan, it is used to prepare koji, a starter for secondary fermentations, using solid-state cultivation, a process thought to have originated in China 3,000 to 2,000 years ago (167). The commercialization of koji dates back to the 13th to 15th century, in Heian and Muromachi periods (167). The long history of extensive use in the food industries placed *A. oryzae* on the FDA list of Generally Recognized as Safe (GRAS) organisms in the United States (168). For making koji, *A. oryzae* spores are spread onto steamed rice. After ~2 days of growth, the resulting *A. oryzae*-rice mixture (koji) is mixed with soybeans or additional steamed rice and water and fermented mainly by yeasts to produce miso, shoyu, and sake (169, 170). The fungus has an important role in the degradation of proteins and starch contained in the raw material for fermentation and contributes to the color, flavor, and aroma of the fermented foods. Starch is converted into simple sugars by a biochemical process called saccharification.

A. oryzae was first isolated from koji in 1876 by H. Ahlburg. It appeared morphologically almost indistinguishable from *A. flavus* (171) and nearly identical when analyzed by DNA/DNA hybridization (172). Unlike *A. flavus*—which produces the toxic and carcinogenic aflatoxins, a serious problem worldwide in agricultural commodities such as maize, peanuts, tree nuts, and cotton seeds (173)—*A. oryzae* is a nontoxigenic species (174). However, using multilocus genotyping and sequencing analysis, *A. oryzae* strains were shown to form a monophyletic clade nested within *A. flavus*, closely related to atoxigenic *A. flavus* strains. This finding suggests that *A. oryzae* may have evolved by domestication from a single lineage of *A. flavus* selected for its particular properties (173, 175). This hypothesis is in agreement with previous studies based on SNPs and evolutionary analyses (168, 174). The close evolutionary relationship is reflected in their genomes, which are nearly identical in size and gene content (176, 177). These two genomes are larger than those of *A. fumigatus* and *A. nidulans* due to the expansion of metabolic gene families that may have been acquired by HGT (13).

Genes in expanded families include those encoding secretory hydrolases, transporters, and primary and secondary metabolism. These latter families represent the most expanded gene classes, and they are involved in the degradation of the vast amount of compounds to which the fungi are exposed during fermentation for *A. oryzae*, or in the fields for *A. flavus*, and in the excretion of toxic compounds (13, 167). The fact that these expanded gene families are present in both *A. oryzae* and *A. flavus* indicates that they expanded before domestication (167). *A. flavus* and *A. oryzae* also share the aflatoxin biosynthetic gene cluster, but in *A. oryzae* the gene cluster sequences contain deletions, frameshift mutations, and base pair substitutions (166, 178). No sequence derived from aflatoxin biosynthetic genes was found in the large-scale collection of expressed sequence tags obtained from cDNA libraries from nine biologically or industrially important cultures of *A. oryzae* (179). This indicated the lack of transcription of the *aflR* gene, which encodes a transcription factor positively regulating expression of aflatoxin biosynthetic genes in *A. flavus*. A comparison of the transcriptomes and proteomes of *A. oryzae* and *A. flavus* when grown

on rice showed a global downregulation of biosynthetic genes for secondary metabolites in *A. oryzae*, and particularly of the aflatoxin pathway (166), explaining its inability to produce the toxin. Interestingly, aflatoxin is toxic to *S. cerevisiae* (180), suggesting that the toxicity loss of *A. oryzae* might have been driven by its cooperation with yeast during fermentation for making sake and/or by selection by humans of nontoxic strains (166). Putative adaptations to a starch-rich diet were also found in *A. oryzae*. Several genes involved in carbohydrate primary metabolism, such as those encoding for α-amylase or maltase glucoamylase involved in the catabolism of starch, were significantly upregulated in *A. oryzae*.

Domestication is an artificial selection process that can leave footprints of selective sweeps in genomes as a reduction in polymorphisms in genomic regions encompassing the selected variants. Selective sweeps have been detected in *A. oryzae*, mainly on genes and pathways involved in primary and secondary metabolism. The most relevant genes within selective sweeps are involved in protein and peptide degradation and in carbohydrate metabolism, including a glutaminase gene that catalyzes the hydrolysis of carbon-nitrogen bonds of l-glutamine to produce glutamic acid, a widely used enhancer of food flavor found in considerable quantities in sake (166). The transition to domestication has often been associated with a relaxation of selective constraints and population bottlenecks (46) leaving footprints in the genomes, such as an excess of nonsynonymous substitutions (often deleterious). Surprisingly, this has not been observed in *A. oryzae*; in contrast, the ratio of nonsynonymous to synonymous substitutions and the percentage of estimated deleterious substitutions are lower in *A. oryzae* than in *A. flavus*, and this observed lack of selective relaxation has been discussed with regard to population genetics and the ecology of domestication events (181).

In conclusion, *A. oryzae* is probably the result of a single domestication event from an atoxigenic lineage of *A. flavus*, which sake makers may have selected as a safe mutant, allowing yeast growth in fermented products (57). In *A. oryzae*, the genomic mechanisms of adaptation during domestication appear to have involved mainly changes in secondary metabolism.

TOWARD THE DOMESTICATION OF THE BLACK TRUFFLE (*TUBER MELANOSPORUM* VITTAD.): A GLIMPSE INTO A 2-CENTURY-LONG SAGA

True truffles are hypogeous fungi belonging to the genus *Tuber* (Ascomycota, Tuberaceae), which contains the most famous and prized edible mushrooms (182). The Piemont white truffle (*Tuber magnatum* Picco) and the Périgord black truffle (*T. melanosporum* Vittad.) are the most valuable, the black truffle being the most anciently cultivated in the Western part of the Mediterranean basin (183). Unfortunately, the production of *T. melanosporum* drastically declined during the 20th century, and there are still difficulties in increasing production, despite a sustained planting effort since the 1970s.

The mutualistic biology of truffles requires their association with a plant for accomplishing their sexual reproduction, and in particular for producing the edible fruiting body that results from a mating event (184, 185). Vegetative life depends on the edification of short-lived structures called ectomycorrhizae that connect mycelia to shrubs or trees from various families (e.g., *Fagaceae*, *Salicaceae*, and *Betulaceae*) (186). In spontaneous truffle grounds, the Périgord black truffle mainly associates with oaks but also with Mediterranean shrubs in the *Cistaceae* (187), while it naturally establishes and fruits in early forest stages during secondary succession, mainly in the opened vegetation called "garrigue" (188).

The worldwide notoriety of *T. melanosporum* has fueled the development of unprecedented planting efforts and the introduction of the species in America, Australia, New Zealand, and other countries. As a consequence, truffle growers stimulated the development of highly inventive, but often empirical, techniques to induce truffle production in a wide range of ecological contexts. However, the obligate association between *Tuber* mycelia and trees of few compatible species, combined with the impossibility of producing fruiting bodies *ex situ*, has made the management of truffle growth complex. When remaining aloof, truffle growers act as blind gardeners who indirectly work on truffles through arboriculture practices (e.g., pruning) and agriculture (e.g., tillage or sowing) management methods, over years, with particularly uncertain outcomes.

Here we present a three-step history of the attempts to cultivate truffles, with a focus on the Périgord black truffle. We first deliver a comprehensive assessment of the 2-century-long empirical approach developed in spontaneous truffle grounds since the beginning of the 19th century, with special attention paid to the diversity of practices. Furthermore, we present the tremendous contribution provided by the agronomic approach developed in the 1970s, based on the use of inoculated plants and irrigation practices. We finally discuss the recent development of a global truffle-growing method that includes the input of metagenomics tools and ecological modeling.

The Early Attempts: Empiricalism and Observation in Spontaneous Truffle Grounds

It is likely that the truffle was first gathered as a wild product, although direct reports are lacking. An understanding of its exact ecology was probably delayed by the inconspicuous nature of its vegetative stage. Truffles were harvested and much appreciated by the Romans, although the identity of the species collected 2 millennia ago remains unclear. Pliny the Elder, for example, stated in his *Naturalis Historia* (*Natural History*) that the most valued truffles came from Africa (making a possible reference to the distantly related genus *Terfezia*) and that truffles were formed "when there have been showers in autumn, and frequent thunderstorms,… thunder contributing more particularly to their development; in some places the formation of them is attributed to waterstorms" (189). Yet empirical methods, as in many uses involving microbes, took into account the subterranean link to trees well before any idea of mycelium or mycorrhizae was reached (Table 1).

The emergence of the concept of mycorrhizae itself is tightly linked with truffles. After the Prussian minister of agriculture commissioned a mycologist and botanist, A. B. Frank, to study the origin of truffles to promote their cultivation, Frank in 1885 described the morphology of the relationship of the fungus with tree roots and coined the word *mycorrhiza* (190, 191). He also pointed out the need for "an underlying limestone," i.e., the clear calcicolous ecology of the fungus. Before and after this discovery, many empirical methods already used inoculations on trees (Table 1), but without an established protocol.

Agronomy Considerations: Controlled Inoculation and Irrigation in Planted Truffle Grounds

The first prerequisite to optimize the chance of successfully producing truffles is the production of seedlings with a mycorrhizal relationship with the right fungus. The first successful attempt to synthesize truffle

Table 1 Two centuries of domestication of the Périgord black truffles and production in France

1564: Alphonso Ciccarello, following the ideas of the Arab philosopher Almadel, suggests inoculating trees with crushed fruiting bodies.
1726: Richard Bradley suggests the same method, but for *Tuber aestivum* (Wulfen) Spreng.
Before 1750: Georges Louis Leclerc, Comte de Buffon, inoculates the roots of adult hornbeams in Montbard (Côtes d'Or) with crushed ascocarps of *Tuber melanosporum* and gets truffles 2 years later.
About 1750: Jean-Pierre-François Ripert de Monclar sows acorns in Saint-Saturnin-les-Apt (Vaucluse) and gets black truffles 10 years later.
1790: Pierre II Mauléon sows acorns (*Quercus pubescens* Willd) in Grand-Ponçay (Vienne) and harvests truffles 10 years later.
Between 1808 and 1815: Joseph Talon has the same idea and sows acorns in his fields at Saint-Saturnin-les-Apt (Vaucluse).
Around 1830: 100 to 200 hectares of artificial truffle orchards in the Vienne department and between 300 and 400 hectares in the Drôme and Vaucluse departments are created.
1847: Auguste Rousseau sows acorns (mainly *Quercus ilex*) in a 7-hectare (ha) area near Carpentras (Vaucluse) in previously cultivated soil and irrigates his orchard. He obtained large harvests reaching 70 kg/ha in the best years.
From 1860: 380,000 ha of eroded and overgrazed lands are reforested in southeast France. The Périgord black truffle naturally occurred in these plantations. The Mont Ventoux and the Lubéron mountain became the largest "truffle orchard" in the world.
1866: Bedel creates the "truffle sylviculture," a sylviculture on poor soils with low tree density allowing small-scale production of Périgord black truffles, probably less than 0.5 kg/ha.
From 1863: Beginning of the *Phylloxera* crisis, which destroyed French vineyards, and then in the late 19th century, disease of the silkworm. Numerous vineyards or fields of mulberry trees were replaced by managed truffle orchards, mainly in southeast France. Acorns were also replaced by seedlings cropped under productive trees or produced in nurseries and more or less having mycorrhizal associations with *T. melanosporum* ascospores, using crushed ascocarps but without soil disinfection. In the Drôme and Vaucluse departments, deep soils are used, allowing production reaching several kilograms per hectare in good years.
1885: Discovery and description of the ectomycorrhizae by Albert Bernard Frank.
1894: Dangeard describes ectomycorrhizae of *T. melanosporum* on *Quercus pubescens*.
1900s: Peak of French truffle production, between 500 and 1,000 tons per year.
1914–1918: First World War. National production decreases.
1920–1939: Production is stabilized around 300 tons.
1939–1945: Second World War. Another decrease in production.
1945–1946: Only 66 tons produced.
1947–1960: Stabilization at around 200 tons per year.
1960–1980: Gradual decrease of production, probably resulting from rural exodus.
1980 to present: Stabilization of production around 50 to 60 tons per year.

ectomycorrhizae was realized by Bruno Fassi, Mario Palenzona, and Anna Fontana in Italy (192–196). In 1970–1971, a collaboration started between these Italian scientists and two French scientists, Jean Grente and Gérard Chevalier (197). In 1972, Grente and Chevalier extended this inoculation technique on a large scale and contributed to the creation of the AGRITRUFFE society, exploiting a know-how license INRA/ANVAR. In 1973, AGRITRUFFE put on the market about 10,000 seedlings mycorrhizally colonized with *T. melanosporum* (198). Nowadays in France, 300,000 seedlings (oaks, hazel, hornbeams, pines, etc.) mycorrhizal with *T. melanosporum* are produced in 18 nurseries, and their quality is controlled by INRA or the CTIFL (Centre Technique des Fruits et Légumes) (199). This large-scale inoculation technique was developed first in France and then spread to Italy and Spain and is now used worldwide. At the same time, many truffle growers still use home-made inoculations.

The development of efficient techniques to control *T. melanosporum* inoculation is not the only agronomic contribution of the past decades. Water management during the first 3 or 4 years following planting is crucial for the seedlings and the mycorrhizae (200, 201). To favor the formation of new roots and good development of the *T. melanosporum* mycorrhizae, it is necessary to irrigate the seedlings during summer and to eliminate weed competition by manual hoeing, soil tilling, or mulching (200, 201).

There is increasing evidence that inoculation of the trees with ascospores throughout the truffle orchard life can increase the production of fruiting bodies. During the first years, such inoculations may allow the renewal of *T. melanosporum* mycorrhizae and decrease the chance of dominance of other ectomycorrhizal (ECM) fungi on roots of the host. Three to 5 years after planting, inoculation with ascospores may enhance sexual reproduction, which may be limited by exclusion of one mating type by the other at the mycorrhizal level (202–204) (see below). The current view is that truffle spores added by dispersal of fruiting body pieces act as male contributors to initiate reproduction and thus fruiting body formation: the so-called truffle-trapping designs, mixing spores with soil, often enhance the production of fruiting bodies (205), although the mechanisms, either genetic or linked to soil disturbance, remain unclear.

The Time for a Global Approach: from Metagenomics to Ecological Modeling

Since the early 1990s, the development of metagenomics tools and the *in situ* use of isotope (e.g., ^{13}C and ^{15}N) labeling experiments has opened up new research perspectives on fungal biology and ecology. The below-ground description of truffle grounds based on the barcoding of ECM root tips provided valuable insights into the understanding of the fungal compartmentation in truffle grounds. During the past decade, the sequencing of root apices has been widely applied to truffle plantations and spontaneous systems to provide comprehensive views of below-ground distribution of truffles in *Tuber aestivum* (206), *Tuber borchii* (207), *Tuber macrosporum* (208), and *T. magnatum* (209) grounds. Molecular tools have been developed that are aimed at determining the distribution of *T. melanosporum* in soils and the abundance of black truffle mycorrhizae within ECM communities in planted orchards (e.g., 5, 210) and in its typical habitat (188), where *T. melanosporum* is part of diversified ECM communities, with clear vegetative affinities for oaks as compared to companion shrubs (*Arbutus unedo*, *Cistus* spp.).

In situ labeling experiments have clarified the nutrition of *T. melanosporum* and answered a controversial question debated in handbooks until the 2010s regarding truffle cultivation. The natural abundance of ^{13}C and ^{15}N in tissues of the host and the fungus (211) reflects that *T. melanosporum* behaves like a true ECM fungus that does not exhibit a saprotrophic strategy during the development of its ascocarps, as speculated by some authors (see reference 183 for a review). Subsequently, a labeling experiment of tree photosynthates unambiguously demonstrated sugar transfers from host leaves to fine roots that act as a conduit and transfer carbon to *T. melanosporum* mycorrhizas and finally to the ascocarp, which accumulates host carbon 200 days after the labeling (212). A soil labeling experiment confirmed that *T. melanosporum* did not exhibit saprotrophic capacity for carbon and that carbon used by ascocarps is mainly provided by the host (213). These discoveries will pave the way for the development of pruning calendars that take into account the periods of carbon storage in the wood of the host (winter) and avoid any harmful management practice in periods of carbon demand by the fungus (summer).

The sequencing of the 125-Mb-long genome of *T. melanosporum* (214) was another essential step on the road to truffle domestication. One of the most striking features of this sequencing was the analysis of the region that governs the fungal sexual cycle, showing that *T. melanosporum* is a heterothallic species with two alternative genes at a single *MAT* locus. The provision of these genes revealed the unique population structure of *T. melanosporum* in planted orchards (202). Indeed, the black truffle shows markedly segregated patterns of

spatial distribution of individuals based on their mating type, with long-lived maternal individuals vegetatively established in truffle grounds and paternal individuals remaining cryptic before fecundation (203), leading to the idea that germinating spores (and not mycelia) may be paternal individuals (185, 215, 216). This finding suggests that competition occurs between mycelia of different mating types at the establishment stage and highlights possible mechanisms explaining the absence of fruit body production in large parts of the orchards. Further research is needed to identify paternal reproductive structures in *Tuber* spp. and to detect the ephemeral dikaryotic structure derived from parental mating.

Toward a Domestication of Truffles?

For a long time, the domestication of truffles has been a critical issue for the economy of rural Mediterranean societies (217), and complete control of the life cycle of the fungus is not achieved yet. It is now a spatial planning challenge for stakeholders in many regions of the world who aim to implement a truffle-based development of landscapes (218). Current harvests of Périgord black truffle in its natural Mediterranean habitat are well below production levels in the early 20th century, perhaps as a result of marked changes in landscapes and rural desertification. Exceptional production levels obtained in plantations outside Europe, mainly in Australia, are promising for those who invest, but these countries are too remote to supply the world demand because of the impossibility of controlling all the factors involved during the truffle biological cycle. However, the unprecedented rhythm of scientific knowledge accumulation and the extremely efficient toolbox developed by laboratories give great hope that the synergy between truffle growers and researchers will lead to increasingly understood and controlled practices in the coming years.

CONCLUSION

Fungi have been used safely in food production, even unconsciously, for thousands of years since early agricultural practices, and there is also a long and notable tradition of wild edible fungi in different cultures all over the world. However, only a fraction of edible mushrooms are eaten, and a minute portion of fungi are used in food processing. Fungal biodiversity is evidently a resource pool for food but also for more value-added uses. Fungi are gaining popularity in the context of a global need for new sources of food, and some species are already exploited to supply new-generation bio-based products in proteins or dietary fibers. Moreover, fungal enzymes have huge potential to produce higher-value products as food and feed ingredients from biological raw materials and agro-industrial waste. In addition, they are valuable as good models for studying the genomic processes of adaptation and domestication, with their often small genomes and experimental tractability (18).

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Biologically Active Secondary Metabolites from the Fungi

54

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INTRODUCTION

Fungi, plants, and bacteria are the major kingdoms of life with well-developed secondary metabolism. About 500,000 secondary metabolites (also referred to as natural products) have been described to date. About 100,000 of these are derived from animals, 350,000 are from plants, and 70,000 are from microbes (1, 2). Roughly 33,500 bioactive microbial metabolites have been described (2). Of these 33,500 microbial metabolites, about 12.5% (4,200) are metabolites of unicellular bacteria and cyanobacteria, 41% (13,700) are products of Actinomycete fermentations, and about 47% (15,600) are of fungal origin (1). Furthermore, the rate of discovery of new fungal metabolites has accelerated significantly in the past two decades relative to the rate of discovery in the actinomycetes, filamentous bacteria that traditionally have been the richest source of microbial natural products (2). This complex and rich secondary metabolism is highly developed in the filamentous Ascomycota and Basidiomycota, while it is underdeveloped in the unicellular forms of the Ascomycota and Basidiomycota and in the Zygomycota, Blastocladiomycota, and Chytridiomycota (Fig. 1). The diversity of fungal species, particularly in the Ascomycota and Basidiomycota, and the accompanying diversification of biosynthetic genes and gene clusters points to an almost limitless potential for metabolic variation. In fact, one can argue that much of the ecological success of the filamentous fungi in colonizing virtually all habitats on the planet is owed to their ability to deploy arrays of secondary metabolites in concert with their penetrative and absorptive life forms. This dependence of the fungi on secondary metabolites to conquer diverse habitats and sustain their existence within them is evidenced by the facts that most species make multiple types of secondary metabolites, their expression is orchestrated with the life cycle and environment, and significant portions of their genomes are devoted to encoding and regulating the production of these products.

Secondary metabolites display an incredibly broad range of biological activities. Some have afforded society-changing benefits, while others are associated with serious, almost intractable problems. This dichotomy is indicative of the diversity of natural products that fungi can produce. Fungal metabolites with the greatest negative impact include mammalian toxins commonly known as mycotoxins. As many as 1,000 fungal compounds have been afforded this label, with the most widely known including aflatoxins, trichothecenes, fumonisins, ochratoxin, cytochalasins, and various indole-terpene tremorgenic compounds (3, 4). Organisms in all domains of life produce toxins, including many that are more toxic than those from fungi, but mycotoxins tend to be more problematic because of their widespread occurrence as contaminants of food for humans and livestock, as well as mold-contaminated indoor environments (5). Understanding mycotoxin chemistry is essential to improving the ability to monitor and reduce the levels of exposure to such compounds. Fungi that produce mycotoxins and phytotoxins associated with crop diseases can cause considerable economic losses and require significant investment in their control. Metabolites that function as phytotoxins and small-molecule virulence factors produced by plant pathogenic species are responsible for many of the damaging effects of plant diseases (6).

On the other hand, many important pharmaceuticals have been discovered through studies of fungal chemistry (Table 1). Natural products continue to be among

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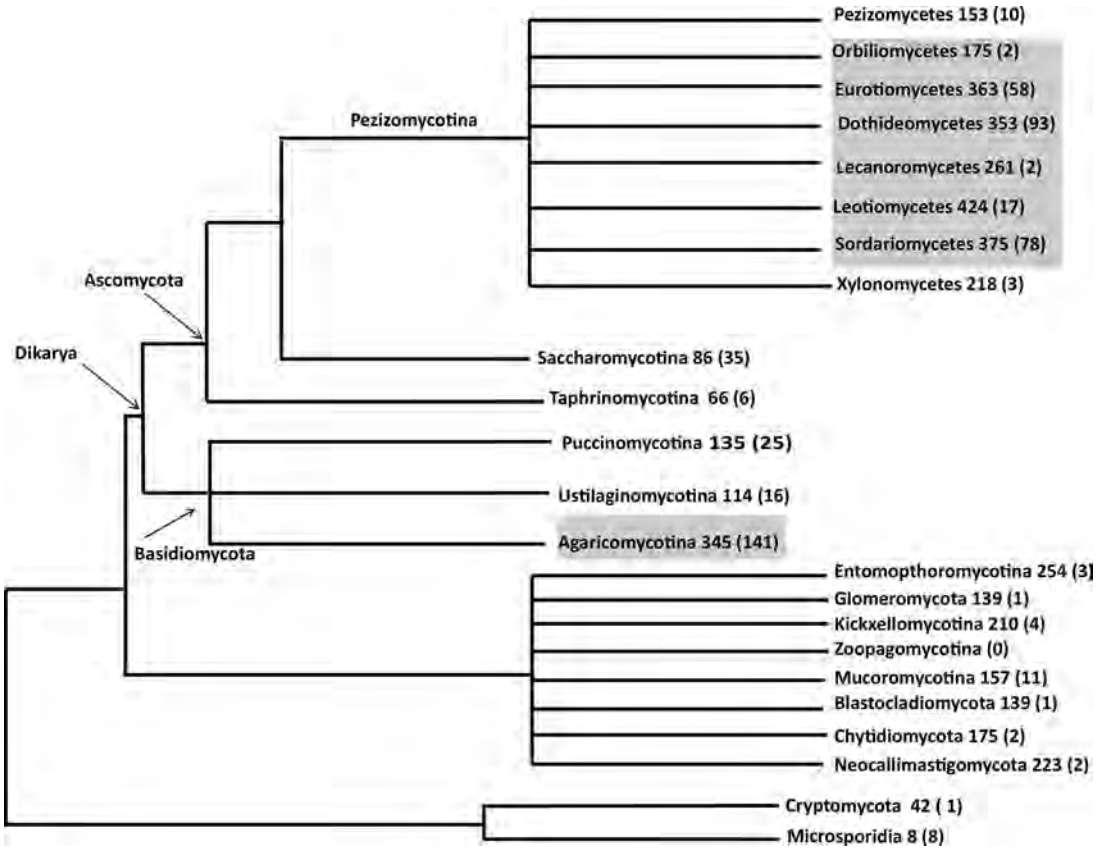


Figure 1 Simplified phylogeny of the phyla and classes of the Fungi. Numbers after phylum or class indicate the average number of secondary metabolite, transport, and catabolism genes recognized by the Clusters of Orthologous Groups of Proteins Classification (KOGs) from sequenced genomes (number in parentheses) of fungi at the Joint Genome Institute's Mycosm Project (January 15, 2016). Taxa shaded in gray are known to produce secondary metabolites with high frequency. Branch length reflects relatedness of taxa.

the most important therapeutic agents and lead compounds in medicine and have been particularly important in the development of effective therapies for cancer, malaria, bacterial and fungal infections, neurological and cardiovascular diseases, and autoimmune disorders (7). Many agricultural chemicals are also natural product-derived (8, 9). Fungi are particularly prolific sources of bioactive secondary metabolites (10) and have contributed in spectacular and indispensable ways to improvements in human and animal health (Table 1). The best known examples are the β-lactam antibiotics, which include penicillins and cephalosporins (Table 1). It is difficult to overstate the tremendous effect these compound classes have had on global health because of their effectiveness against bacterial infections. Moreover, the success of penicillins effectively spawned the development of major technological advances in microbiology, chemistry, biochemistry, and engineering and contributed in many ways to the establishment of the modern pharmaceutical industry. Many other fungal metabolites with valuable activities are known (Table 1), and further examples are incorporated into the discussion below.

One ongoing question regarding the dimensions of fungal biodiversity relates to judging the potential for discovery of even more novel and useful secondary metabolites, as well as the likelihood of further applications for those that are already known. Often one reads provocative statements such as "To date, fewer than 7% of the more than 1.5 million species of fungi thought to exist have been investigated for bioactive components" (11). Such statements are based on the number of metabolites described in the literature and the number of species from which those metabolites have been reported (12). Ultimately, no one really knows what percentage of the total number of species has been tested for biologically active compounds, but it is in all probability much higher. Our experience and

Table 1 Fungal metabolites that have been developed into pharmaceutical, agrochemical, and cosmetic products

Metabolite	Source species	Structural classification	Commercial product(s)	Mode of action	Indication(s)
Penicillins G and V	*Penicillium rubens*, *Penicillium chrysogenum*	Nonribosomal peptide	Benzylpenicillin and phenoxymethylpenicillin; 6-aminopenicillanic acid derived from penicillin G is acylated to make semisynthetic penicillins with diverse side chains, e.g., piperacillin, amoxicillin, and ampicillin	Interferes with biosynthesis of cell wall peptidoglycans	Deep-seated infections caused by Gram-positive and some Gram-negative bacteria
Cephalosporin C	*Acremonium chrysogenum*	Nonribosomal peptide	The 7-amino cephalosporanic acid core is used to produce numerous derivatives, including cephalothin, cephalexin, cephradine, and cefadroxil	Interferes with biosynthesis of cell wall peptidoglycans	Treatment of Gram-positive and some Gram-negative bacterial infections
Pleuromutilin	*Clitopilus passeckerianus*, other *Clitopilus* spp.	Diterpene	Precursor for the partial chemical synthesis of topical antibiotic retapamulin (Altabax) and animal antibiotics tiamulin and valnemulin (Econor)	Targets peptidyl transferase center of bacterial ribosome	Topical treatment of infections from Gram-positive bacteria, treatment of Gram-positive bacterial infections in livestock
Fusidic acid	*Acremonium fusidioides*	Triterpene	Usidin, Fucidin, Fucicort, Fucibet, Taksta, and others	Prevents translocation of the elongation factor G from the ribosome	Topical antibiotic for Gram-positive bacterial infections including methicillin-resistant *Staphylococcus aureus*; under development for prosthetic joint infections
Strobilurins A–D and other strobilurins and oudemansins	*Strobilurus tenacellus*	Polyketide with benzoyl CoA starting unit	Templates for synthetic family of β-methoxyacrylate strobilurins including azoxystrobin, fenamidone, fluoxastrobin, kresoxim methyl, and trifloxystrobin	Targets the mitochondrial cytochrome b and interferes with respiratory electron transport	Fungicides for broad range of plant pathogenic fungi
Griseofulvin	*Penicillium griseofulvum*, *Penicillium aethiopicum*, *Penicillium Coprophilum*, and other *Penicillium* spp.	Halogenated polyketide	Fulcin, Fulsovin, Grisovin, and others	Inhibits mitosis by binding to tubulin and preventing tubulin polymerization	Treatment of fungal infections of the skin, hair, and nails
Pneumocandin B_0	*Glarea lozoyensis*	Nonribosomal peptide acylated to polyketide	Precursor of the partial chemical synthesis of caspofungin (Cancidas)	Inhibits β-1,3 glucan synthase and cell wall biosynthesis	Treatment of systemic fungal infections
FR901379	*Coleophoma cylindrospora* (= *C. empetri*)	Nonribosomal peptide acylated to fatty acid	Precursor of the partial chemical synthesis of micafungin (Mycamine, Fungiguard)	Inhibits β-1,3 glucan synthase and cell wall biosynthesis	Treatment of systemic fungal infections
Echinocandin B	*Aspergillus pachycristatus* and other *Aspergillus* spp. of section *Nidulantes*	Nonribosomal peptide acylated to fatty acid	Precursor of the partial chemical synthesis of anidulifungin (Eraxis)	Inhibits β-1,3 glucan synthase and cell wall biosynthesis	Treatment of systemic fungal infections

(Continued)

Table 1 Fungal metabolites that have been developed into pharmaceutical, agrochemical, and cosmetic products *(Continued)*

Metabolite	Source species	Structural classification	Commercial product(s)	Mode of action	Indication(s)
Enfumafungin	*Hormonema carpetanum*	Triterpene glycoside	Precursor of the partial chemical synthesis of experimental drug Scy-078 that is in phase II clinical trials	Inhibits β-1,3 glucan synthase and cell wall biosynthesis	Treatment of systemic fungal infections
Lovastatin (monacolin K)	*Aspergillus terreus*, *Monascus purpureus*, also occurs in basidiomata of *Pleurotus ostreatus*	Polyketide, product of two highly reducing polyketide synthases	Mevacor Simvastatin (Zocor, Simvacor, Simvastin, and other brand names) is made by semisynthesis from lovastatin	Inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase, prevents conversion of mevalonate into cholesterol	Treatment of hypercholesterolemia to reduce risk of cardiovascular disease
Compactin (Mevastatin)	*Penicillum citrinum*, *Penicillum solitum*, and other *Penicilium* spp.	Polyketide, product of two highly reducing polyketide synthases	Although recognized as the first statin, never approved as a drug. Used for biotransformation or partial chemical synthesis from mevastatin (Pravachol, Selektine, Lipostat, and other brand names)	Inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase, prevents conversion of mevalonate into cholesterol	Treatment of hypercholesterolemia to reduce risk of cardiovascular disease
Cyclosporin A	*Tolypocladium inflatum*	Nonribosomal peptide	Cyclosporine A (Sandimmune, Neoral, Restasis, Gengraf, and other names)	Binds to cyclophilin A, resulting in calcineurin inhibition, which activates transcription of interleukin 2. Also inhibits lymphokine production and interleukin release	Prevention of organ transplant and tissue graft rejection
Mycophenolic acid	*Penicillium brevicompactum* and other *Penicillium* spp.	Meroterpenoid	Used to produce mycophenolate mofetil (Cellcept), mycophenolate sodium (Myfortic)	Inhibits inosine monophosphate dehydrogenase (IMPDH). T and B lymphocytes are heavily dependent on the activity of IMPDH to make DNA to proliferate	Prevention of organ rejection following kidney, liver, or heart transplants
Myriocin (ISP-I)	*Isaria sinclairii*	Amino acid lipid	Structural template for synthesis of fingolimod (Gilenya)	Activated form binds to extracellular G protein-coupled receptors and prevents release of lymphocytes that enter the CNS	Treatment of multiple sclerosis
Ergotamine	*Claviceps purpurea*, *Claviceps fusiformis*, and *Claviceps paspali*	Prenylated nonribosomal peptide	Ergotamine tartrate (Ergomar, Migril, and others), dihydroergotamine mesylate (Migranal), or combined with caffeine (Cafergot, Cafetrate, and other names), dihydroergotamine (DHE 45)	Neurotransmitter mimic, agonists of 5-OH tryptamine (5-HT2) receptor 1B, dopamine, norepinephrine, and serotonin receptors	Vasoconstrictor used as antimigraine agent, also combined with belladonna and phenobarbital for relief from menopausal hot flashes

Ergometrine (ergonovine)	*C. purpurea*, *C. fusiformis*, and *C. paspali*	Prenylated nonribosomal peptide	Ergometrine maleate (Ergotrate, Ergovin, and others); Methylergometrine is a synthetic version, sometimes combined with oxytocin (Syntometrine) for veterinary usage	Serotonin antagonist, activating (or blocking) 5-HT2 receptors	Treatment of postpartum haemorrhage
			Used for partial chemical synthesis of lysergic acid diethylamide, LSD-25, LSD (Delysid)		Hallucinogen, treatment of psychosis and depression
Ergocryptine	*C. purpurea*, *C. fusiformis*, and *C. paspali*	Prenylated nonribosomal peptide	Partial chemical synthesis of bromocriptine (Parlodel and other names)	Agonist of dopamine D_2 receptors and various serotonin and adrenergic receptors	Treatment of reproductive disorders, e.g., galactorrhoea, prolactindependent mammary carcinoma, amenorrhoea, acromegaly or anovulation, complications, e.g., osteoporosis. Bromocriptine is also used in the treatment of Parkinson's disease
Mizoribine	*Penicillium brefeldianum*	Imidazole nucleoside	Bredinin and other names	Competitive inhibitor of IMPDH	Immunosuppressant used for renal transplants in Japan, Korea and China
Kojic acid	*Aspergillus oryzae*. *Aspergillus tamarii*, *Aspergillus flavus*	Pyrone derived from glucose	Often formulated as kojic dipalmitate to increase stability and shelf life	Inhibits tyrosinase and suppresses melanogenesis	Antioxidant in cosmetic products, used to lighten skin color and treat abnormal hyperpigmentation
PF1022A	*Rosellinia* sp.	Nonribosomal peptide	Emodepside is the bis-paramorphonyl-derivative of PF1022A	Disrupts target nematode neuromuscular function due to interactions with the latrophilin-like HC110-R G-protein coupled and SLO-1 potassium channels	Combined with praziquantel, another anthelmintic drug, to treat roundworm, hookworms, and tapeworms in cats as Profender
Fumagillin	*Aspergillus fumigatus*	Meroterpenoid	Bicyclohexyl ammonium fumagillin	Inhibits methionine aminopeptidase2 (MetAP2)	Control of nosema disease in honey bees caused by *Nosema apis*
Gibberellic acid (GA)	*Fusarium fujikuroi*	Diterpene	Produced by fermentation of *F. fujikuroi*	GA signaling promotes plant growth by overcoming DELLA-mediated growth restraint	Plant growth hormone for plant tissue culture and applications for certain high-value crops
α-Zearalanol (Zeranol)	*Fusarium* spp., including *Fusarium culmorum*, *Fusarium sporotrichioides*, *Fusarium equiseti*, and *Fusarium semitectum*	Polyketide, product of a highly reducing and a nonreducing polyketide synthase	Produced by fermentation of zearalenone, then hydrogenation to form α-zearalanol, formulated as Ralgro implants	An estrogen mimetic that binds to mammalian estrogen receptors α (ER α) and ER β	An anabolic agent used to increase the rate of weight gain and feed conversion in cattle in North America

that of our colleagues who have worked in the pharmaceutical and agrochemical industries tells us that far more species have been surveyed for secondary metabolites of interest over the decades in discovery screening programs than the literature would indicate, because inactive, strongly toxic, redundant, or unproductive fermentation extracts and products are rarely pursued, let alone published or otherwise documented (13, 14). Recognition of already known compounds in fungal fermentations—a process referred to as dereplication (15, 16)—is a vital step in natural product discovery processes. The high frequency of encounters with known compounds in screening programs represents a substantial resource cost that generally affords little value. Although improvements in the efficiency of dereplication processes continue to develop with advances in relevant analytical technologies (16), the costs in time and effort associated with "rediscovery" of known metabolites pose a significant obstacle to natural product discovery efforts. Such findings generally result in disinterest, and certainly not in publication. Other phenomena, such as compound decomposition or loss during separation efforts or the possibility that mixtures may exert effects not detected in purified/individual metabolites, may ultimately prevent identification of secondary metabolites that may be present.

Fermentations of most strains in major service collections have probably been examined for biologically active compounds at some level (17), yet most of this work likely is undocumented. For example, in the past, pharmaceutical companies would routinely buy strains from major service collections when the collection staff made routine transfers of their stock collections. It is impossible to know how many fungi have really been screened or how many compounds have been produced, detected, purified, and partially or fully characterized but then discarded for lack of potency and target specificity in the assays being employed for screening. To complicate such speculation even further, the window of desired biological activity in a given screening program often may be quite narrow, e.g., inhibition of bacterial type II topoisomerases, and negative data from these efforts are seldom considered worthy of publication. What is clear is that the numbers of possible applications for fungal metabolites in medicine, agriculture, cell biology, and consumer products are limitless and constantly evolving. Even well-known metabolites, previously discarded for one application, e.g., an antibiotic, can suddenly become recognized for new and exciting biology. In our opinion, exploration of fungal chemistry with the aim of finding new applications is likely to be far more productive than surveying what one believes are new fungi using dated screening methods, e.g., growth inhibition of typical model strains of human pathogens, and certainly more productive than simple chemical screening with no biological detection method at all.

No example illustrates the unforeseen value of "ordinary" fungal metabolites better than mycophenolic acid (MPA) (Fig. 2a, Table 1) (18). MPA was discovered by Bartolomeo Gosio from *Penicillium brevicompactum* in 1893, and it has the distinction of being the first antibiotic to be purified in crystalline form (19). It inhibited the growth of *Bacillus anthracis*, but quantities were insufficient for further investigation. Its medical application as an antibiotic was later investigated by C. L. Alsberg and O. M. Black in 1912 (20) and by Howard Florey's team at Oxford in 1946 (21), but it was discarded as a potential antibiotic due to its toxicity. During the 1960s and 1970s, MPA was recognized to have remarkable biological activities against viruses and certain tumor types, and its mode of action as an inhibitor of inosine 5′-monophosphate dehydrogenase, an enzyme required for the biosynthesis of guanine, was elucidated. Depletion of the cellular guanine derivative pool, therefore, accounted for many of MPA's effects. These mechanistic studies led to explorative studies of MPA as an oral treatment for psoriasis (22). Inhibition of this critical metabolic pathway in more susceptible human T and B lymphocytes was later exploited in the development of the immunosuppressive agent mycophenolate mofetil, marketed under the names CellCept (Hoffmann LaRoche) and Myfortic (Novartis).

Beyond its impact as the precursor for this important new class of immunosuppressant drugs, MPA illustrates the current transformation underway in modern natural product research. The complete biosynthetic pathway has been characterized in two producing fungi, *P. brevicompactum* (23, 24) and *Penicillium roqueforti* (25). MPA production is catalyzed by interaction of MpaA, a prenyl transferase, with a polyketide synthase (PKS), MpaC, producing 5-methylorsellinic acid, and MpaDE, which is a newly recognized hybrid enzyme,

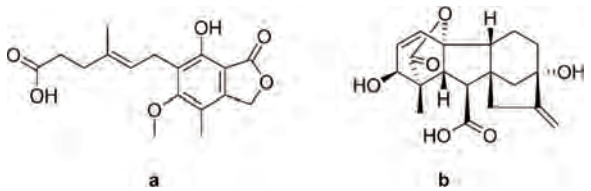


Figure 2 Structures of (**a**) mycophenolic acid and (**b**) gibberellic acid.

fusing a cytochrome P450 domain and a hydrolase domain, catalyzing the production of 5,7-dihydroxy-4-methylphthalide (23). By reconstituting the entire gene cluster in a heterologous host, *Aspergillus nidulans*, the functions of the genes encoding and regulating MPA biosynthesis have been characterized, and the feasibility of employing *A. nidulans* as a microbial cell factory for MPA has been established (23).

This review will briefly summarize general aspects of fungal secondary metabolism and recent developments in our understanding of how and why fungi make secondary metabolites, how these molecules are encoded, and how their encoding genes are distributed across the Fungi. Readers seeking in-depth and detailed works on fungal metabolites and their biosynthesis should consult information from more comprehensive works (3, 26–31) and a new database on functionally characterized secondary metabolite gene clusters (32). In addition, we will illustrate the breadth of fungal secondary metabolite diversity by highlighting recent information on the biosynthesis of some of the most important fungus-derived metabolites that have contributed to human health and agriculture, as well as those that have negatively impacted crop production, food distribution, and human-built environments.

WHAT ARE SECONDARY METABOLITES AND THEIR FUNCTIONS?

Classical definitions of secondary metabolites convey the idea that these compounds are not essential for growth or survival of the producing organism. While primary metabolism is generally shared by most organisms, a general characteristic of secondary metabolites is that they have a biased phylogenetic distribution due to multiple factors that have driven extinction and diversification of their encoding genes and gene clusters along narrower evolutionary lineages. In the laboratory, genes encoding the primary catalytic enzymes for secondary metabolite synthesis can often be silenced by gene disruption with relatively minor effects on vegetative growth or sporulation (33–35). However, the essentiality of secondary metabolism is frequently contextual, and some secondary metabolites that appear to fall into this category are indispensable to survival in nature, e.g., pigments, sunscreens, siderophores, and pheromones. What is clear is that their importance to the organisms that produce them is underestimated because of their incomplete inventory and their unidentified or misunderstood functions. It has been argued that secondary metabolites and other small bioactive molecules of biogenic origin are so vital to the organisms that produce them that they should form the fourth pillar of "the central dogma of biology" along with DNA, RNA, and proteins (36). The line between secondary and primary metabolism can be blurry, and sometimes primary metabolites may behave like secondary metabolites, e.g., oxalic acid (37–39). Depending on their perceived essentiality and functions, secondary metabolites can include simple molecules derived directly from primary metabolism, such as alcohols, sugars, and organic acids. As an example, the discovery and characterization of quorum-sensing molecules and related systems in bacteria, and more recently in yeasts, have changed popular notions of the roles of low-molecular-weight compounds in microbial communities (40).

Fungi can release their secondary metabolites from cells as volatiles, excrete them into the environment, or incorporate them into structural elements of the cell, or they can remain cell-bound. The term "extrolites" has been suggested as an alternative descriptor for such compounds based primarily on the fact that their biological functions are assumed to be outwardly directed (41), but because the functions and sites of cellular biosynthesis for so many remain unknown, because some have self-directed functions, and also for the sake of continuity, we prefer to employ the classical term "secondary metabolite."

From a chemical standpoint, secondary metabolites are on one end of a continuum of metabolites and are categorized as compounds biosynthesized by pathways that employ primary metabolites as building blocks to assemble more complex molecules, such as polyketides, terpenoids, nonribosomal peptides, nuclear-encoded ribosomal peptides, and molecules of mixed biogenic origin arising from hybrid pathways. These categories, representative examples of each, and related recent developments are discussed in separate sections below. Thousands of secondary metabolites are known and catalogued in various compendia and databases (12). Many of them have been encountered and identified because of their association with a biological activity or phenomenon detected outside of laboratory settings (e.g., mycotoxins, phytotoxins, pigments). In a few groups of fungi, secondary metabolite profiles of fungal reproductive structures or cultures have been characterized and correlated with taxonomic and phylogenetic classifications (42–46). However, much of our knowledge of such chemistry has arisen through extensive screening programs conducted in the pharmaceutical and agricultural industries, as well as in academic laboratories, in search of small molecules with potentially useful effects. Bioassays of various kinds are typically

used to guide the isolation of secondary metabolites from complex fermentation extracts using various chromatographic methods, and they are identified using spectroscopic techniques, most commonly nuclear magnetic resonance, mass spectrometry, and X-ray crystallography. These tools, along with databases that store spectral and other physical data, have grown rapidly in their capabilities, greatly expanding the ability to purify and identify such compounds and to detect and quantitate them in various samples.

Secondary metabolites that have been reported from both fungi and plants are not common. Of course, differences in secondary metabolism would be expected from these two domains of eukaryotic organisms due to differences in their basic metabolism and evolutionary origins of their respective biosynthetic pathways. Some of these differences stem from initial steps in biosynthetic processes that influence the prevalence of certain precursors. For example, decarboxylation of amino acids is much more common in plants than in fungi, affording amine precursors that are ultimately incorporated into many classes of plant alkaloids that have not been encountered among the fungi. The phenylpropanoid pathway so common among plants is not as prevalent among fungi. Similarly, secondary metabolic differences also occur between fungi and other microorganisms. For example, polyketide production in fungi generally involves poly-1,3-dicarbonyl precursors generated from acetyl and malonyl CoA. Bacteria also produce polyketides but can also utilize propionyl and butyryl CoA in such processes, leading to different kinds of end products. The dominant type of PKSs in plants are type III PKSs, such as chalcone synthase, a well-known plant type III PKS that catalyzes the first committed step in flavonoid biosynthesis.

Some secondary metabolites are made by both plants and fungi, such as gibberellins, diterpenoid plant hormones that can also be produced by certain fungi, albeit via different enzymatic pathways. The intensively studied gibberellic acids (GAs) (e.g., Fig. 2b) represent the best known unequivocal case of convergent secondary metabolites between plant-inhabiting fungi and plants. They are based on the tetracyclic *ent*-gibberellane terpenoid skeleton and are produced by plants, as well as the plant pathogen *Fusarium fujikuroi*, and are occasionally reported from other fungi (47, 48). In plants, the GAs are a major family of hormones that promote and regulate plant growth. The commercial manufacture of GAs via fungal fermentation has enabled the development of these natural plant hormones into high-value plant growth regulatory hormones for plant tissue culture, horticultural, and biotechnological applications (Table 1). In *F. fujikuroi*, the GA gene cluster contains a pathway-specific geranylgeranyl diphosphate synthase gene (ggs2) that seems to form geranylgeranyl diphosphate exclusively for GA biosynthesis. In addition, a bifunctional CPS/KS (copalyl diphosphate synthase, CPS/*ent*-kaurene diphosphate synthase, KS)-encoding gene, *cps/k*s, catalyzes the cyclization steps to *ent*-kaurene, and four P450 monooxygenase-encoding genes and a desaturase gene carry out the oxidative steps and introduce a double bond, respectively.

The question of whether endophytic fungi may biosynthesize secondary metabolites in physiologically relevant quantities in living plants has received considerable attention. Ergot alkaloids are mycotoxins characteristically produced by fungi in the Clavicipitaceae that form biotrophic infections of grasses and some species of the *Convolvulaceae* (morning glories and relatives). The fungi and their metabolites can increase host plant resistance to herbivores and cause toxicity to consuming livestock (49). Some species of *Convolvulaceae* are infected by endophytic clavicipitaceous fungi (*Periglandula* spp.) that produce high concentrations of ergot alkaloids in seeds—up to 1,000-fold greater than in infected grasses. Swainsonine is an indolizidine alkaloid and is the toxic component that causes livestock poisoning upon consumption of locoweeds (*Astragalus*, *Oxytropis*, and *Swainsona* spp. of the *Fabaceae*) (50). It can also be found in certain species of *Ipomea* and *Turbina* (*Convolvulaceae*) and *Sida carpinifolia* (*Malavaceae*). In a manner analogous to ergot alkaloid production in grasses, the swainsonine in plant tissues results from infection by seed-transmitted endophytic fungi of the genus *Undifilum* (Dothideomycetes, Pleosporales) that produce swainsonine in living tissues of locoweeds. Swainsonine production in plants of the *Convolvulaceae* appears to be associated with an endosymbiotic fungus of the order Chaetothyriales (270).

Biosynthesis of secondary metabolites may be tightly regulated. Their expression is often triggered by chemical or environmental stimuli and coordinated with the development and morphogenesis of the producing organism (51–54). For example, early studies showing that certain bioactive secondary metabolites were strongly associated with *Aspergillus* sclerotia have been supported by more recent investigation of tissue-specific regulation of gene expression in sclerotia versus mycelia (52). Even so, details regarding their functions in nature remain elusive. In some cases, expression may be stimulated or repressed by association with other organisms (55, 56). Because some secondary

metabolites are products from pathways that are expressed in a particular context or life stage, our perception of secondary metabolism in the laboratory depends on whether the context-dependent stimulus or life stage can be artificially replicated and whether pathways are expressed under typical laboratory environmental and nutritional conditions. Some products are easily produced in culture and thus are repeatedly encountered, while other pathways remain silent in artificially cultured strains. The long history of fermentation technology has documented that growing strains under varied nutrient and aeration regimes can profoundly change metabolite compositions and titers (57–59), e.g., plant-derived media versus defined media (60) or liquid versus solid media (61). Experimental efforts to capitalize on these phenomena have remained largely empirical. This knowledge gap has been a key rate-limiting factor in the identification of the natural functions of secondary metabolites, as well as in the discovery of new applications of secondary metabolites in medicine and agriculture.

Secondary metabolite gene clusters are the product of selection of complex traits over millions of years of evolution. Mechanisms contributing to the diversification of the end products include gene duplication, fusion, neofunctionalism, transposition, recruitment and loss of additional genes, and in some cases, horizontal gene transfer (HGT) (62, 63). During the past decade, apparent discontinuous distributions of many secondary metabolite pathways has been attributed to HGT, or in other words, the acquisition and stable inheritance of foreign genes by a recipient organism outside of the process of karyogamy. Although HGT is prevalent in prokaryotes and was responsible for the origin of eukaryotic mitochondria and plastids, through endosymbiosis, the degree to which HGT has impacted eukaryotic evolution remains controversial. In principle, the introduction of foreign DNA into fungal genomes might occur during unicellular or early developmental stages, when their nuclear DNA is relatively exposed to potential sources of HGT, but as of yet, the mechanisms of these proposed eukaryote HGT processes remain unknown. Nonetheless, if genes for a novel metabolic process move by HGT, they endow the recipient organism with the possibility of acquiring a new selective advantage that might promote the survival of the recipient and therefore also of the genes. Clustering of biosynthetic, regulatory, and resistance genes would favor dispersal of entire pathways. Evidence for HGT can be particularly strong because the putatively transferred gene carries a nucleotide composition of the donor genome at the time of transfer from the donor organism and is often easily recognized in the case of bacterial-to-eukaryotic HGT. For example, the partially reducing, 6-methylsalicylic acid (6-MSA)-type polyketide synthases that are widespread across the Fungi may have originated from Actinomycete ancestor proteins (64). However, in the case of hypothetical interascomycete transfers, the direction of transfer is usually not apparent.

The principal method for identification of HGT is phylogenetic incongruence as measured by strong statistical conflict between the phylogenies of the putatively transferred gene and of the background genes of the organism. These types of analyses are highly dependent on the availability of genomes and sampling of species. What at first seems to be HGT of a biosynthetic cluster between phylogenetically distant species may later be recognized as congruent as new gene cluster orthologues from newly sequenced genomes become available. An especially high-profile example was the report of a putative HGT of the sterimatocystin gene cluster from *A. nidulans* (Eurotiales) to *Podospora anserina* (Sordariales) (65). The authors of this report verified that sterigmatocystin genes were transcribed, but they never tested whether *P. anserina* produced sterigmatocystin. Subsequently, we have independently observed sterigmatocystin production in at least one freshly isolated strain of *P. anserina* (J. Gloer, G. Bills, unpublished data). A broader analysis of fungal genomes inferred that the donor organism of the *P. anserina* sterigmatocystin gene cluster may have been a species in the Dothidiomycetes, e.g., *Rhytidhysteron rufulum* (66).

Neither of these reports took into account the widespread reports of sterigmatocystins or their biosynthetic analogues in other fungi belonging to the Sordariomycetes, including *Chaetomium* spp. (Sordariales) (67), *Aschersonia coffeae* (Hypocreales) (68), *Monicillium nordinii* (Hypocreales) (69, 70), *Calcarisporium arbuscula* (Hypocreales) (71), or other fungi in the Dothidiomycetes, including an unidentified species in the Microthyriaceae (72). Furthermore, orthologues of genes in aflatoxin-sterigmatocystin gene clusters have been recently detected in the genomes of the causal agent of chalkbrood disease of bees, *Ascosphaera apis* (Onygenales) (73), the soil fungus *Ochroconis mirabilis* (Venturiales) (74), and the tomato pathogen *Cladosporium fulvum* (Capnodiales) (75). Thus, sterigmatocystin-aflatoxin pathway genes appear to be more widely distributed across the Dothidiomycetes, Eurotiomyctes, and Sordariomycetes than previously thought (67), and the gaps will likely continue to be filled in as more genomes are sequenced. One wonders

if new phylogenetic reconstructions based on the sterigmatocystin-aflatoxin pathway genes and associated housekeeping genes will eventually approach congruency once the analyses include data from the gene clusters from all of these fungi and others that have yet to be discovered. For the time being, our preference is to treat these putative discontinuous distributions as HGT hypotheses because unequivocal evidence is lacking and relatively few fungal genomes have been fully sequenced. As more fungal genomes become available, we predict that alternative evolutionary models will emerge that will explain these reticulate patterns of secondary metabolite distributions.

The events leading to secondary metabolite diversification are undoubtedly linked with speciation and niche radiation, but how selective pressures determine the genetic origins and distribution of these pathways, and their feedback on species fitness, remains obscure (76). Some secondary metabolites are critical to the ecological success of the Fungi because they mediate micronutrient acquisition and transport, virulence, intra- and interspecies communications, defensive mutualisms, protection against abiotic stress, and reproductive development and form pigments, among other functions (77). A puzzling aspect of the prevalence of secondary metabolism is that only a tiny fraction of secondary metabolites have a demonstrated effect that can be linked to a competitive advantage to the producer (78, 79). Design and interpretation of laboratory experiments to identify the natural function of a secondary metabolite discovered out of its natural context are notoriously difficult (79–81). Therefore, progress in dissecting the functions of microbial chemistry in nature has been limited to relatively few systems, such as plant and animal defensive mutualisms and host-pathogen relationships. Classic hypotheses regarding interference competition or defense have contributed explanations of the vast chemical repertoire of fungi and other microorganisms by incremental and reciprocal adaptations and counteradaptations between fungi and their symbionts, parasites, hosts, and predators, shaped by mutual selection (82–86). These hypotheses have underpinned fungal metabolite discovery projects since the discovery of the first antibiotics in the late 19th century and continue to guide modern fungal natural products research within the pharmaceutical and agrochemical industries.

Concepts of some secondary metabolites as agents of chemical defense or offense are further supported by observations that antibiotic resistance genes are common in nature and that the organisms that produce them often have complex self-resistance mechanisms (87–89). As in bacterial secondary metabolite gene clusters, evidence that fungal gene clusters can encode auxiliary target proteins to evade self-toxicity from their own metabolites is accumulating. One of the best examples of self-resistance genes for a fungal secondary metabolite occurs in the pathways for the hydroxymethylglutarate (HMG)-CoA reductase inhibitors, lovastatin and compactin (Table 1). These metabolites are weakly antifungal because they block production of mevalonate, which in turn affects steroid and terpene synthesis. The lovastatin and compactin gene clusters in the producing fungi carry an ancillary HMG-CoA reductase gene (*Aspergillus terreus*, *lvrA*; *Monascus pilosus*, *mokG*; *Penicillium citrinum*, *mlcD*) which confers resistance to the pathway product by producing the mevalonate precursor during compactin and lovastatin biosynthesis (90, 91). A strain of *P. citrinum* with mutated *mlcD* was 200-fold more sensitive to compactin and produced lower ergosterol levels than the parent strain (90). *Aspergillus fumigatus* produces the epipolythiodioxopiperazine gliotoxin from a bimodular nonribosomal peptide synthetase (NRPS) as an immunosuppressive virulence factor during pathogenesis (92). Disruption of the *gliT* gene, encoding a gliotoxin reductase, in the biosynthetic gene cluster resulted in reduced production of gliotoxin and sensitization of the mutants toward gliotoxin (93). The immunosuppressive metabolite MPA, discussed above, potently inhibits inosine-5′-monophosphate dehydrogenase (IMPDH). Characterization of the MPA biosynthetic gene clusters from *P. brevicompactum* and *P. roqueforti* revealed an auxiliary copy of an IMPDH-encoding gene (*mpaF*) embedded within the cluster that is resistant to MPA (24, 25). Self-toxicity also may be mitigated by tight regulation of product biosynthesis during the critical stages of the life cycle (53), by transport proteins specific for biosynthetic pathways, and by product-specific trafficking in specific cells and intracellular compartments (89, 94).

RUDIMENTARY SECONDARY METABOLITES: EVOLUTION OF THE FIRST BIOACTIVE SMALL MOLECULES

The origins of secondary metabolites are likely to coincide with the origins of self-replicating biological molecules (95–97). One can easily imagine how simple amino acids reacted with each other and other organic molecules to form new molecules that interacted with the earliest macromolecular processes; when these rudimentary secondary metabolites exerted positive effects on macromolecular processes and ultimately on

reproduction of the cell, they were retained as a new cellular process. Many consider that the early chemical and structural interaction sites of primordial amino acids, which gradually evolved into polypeptides, still retain some level of that specificity today (98). Indeed, the antagonistic activity of antibiotics, among their other varied small-molecule interactions, could simply be one facet of their primordial origins. And while not constituting proof of the role of secondary metabolites in nascent biochemical evolution, their ubiquity is consistent with the belief that bioactive small molecules have been critical to forging diversification of all organisms throughout molecular history (36, 96).

Many of these rudimentary metabolites (e.g., Fig. 3) are amino acid derivatives or by-products of amino acid biosynthesis. Given the importance of amino acids in cell composition and function and as building blocks of many secondary metabolites, the nonribosomal peptides may be the oldest secondary metabolites (97). One of the simplest fungal secondary metabolites is hadacidin (*N*-formyl hydroxyaminoacetic acid) (Fig. 3a), a compound found in *Penicillium frequentans* and other *Penicillium* spp. (99). Its name is based on its inhibition of human adenocarcinoma (100, 101). Furthermore, it potently inhibits plant growth (102). Hadacidin inhibits purine biosynthesis by inhibition of adenylosuccinate synthetase. Isotope labeling studies indicated that it is formed by *N*-oxygenation of glycine to yield *N*-hydroxyglycine, followed by *N*-formylation to give the hydroxamic acid. Another intriguing example of primitive secondary metabolites is provided by cyclic dipeptides (CDPs; diketopiperazines or dioxopiperazines) formed by the linkage of two amino acids. CDPs have been suggested to be among the most ancient classes of signaling molecules (98). These compounds are ubiquitous in biological systems and have even been found in extraterrestrial meteorites (103). In abiotic systems, CDPs are produced from unprotected linear dipeptides, and once formed, they remain relatively stable. The lack of free C- and N-terminal groups enables CDPs to resist human digestion, so they are attractive templates for the development of protein-based drugs.

Aside from spontaneous formation in primordial stews, they are produced by two major biosynthetic routes: as products of dedicated NRPSs (see below) or, more interestingly, by a recently discovered enzymatic process employing cyclodipeptide synthases (CDPSs) (104–106). CDPSs commandeer aminoacyl-tRNAs and employ them in the formation of a cyclodipeptide link from two aminoacyl transfer RNAs using a ping-pong mechanism that transfers an aminoacyl moiety of the first aminoacyl tRNA onto a conserved serine residue, yielding an aminoacyl enzyme. At the same time, CDPSs divert the flow of loaded tRNAs away from the ribosomal machinery (Fig. 4). The covalently bound

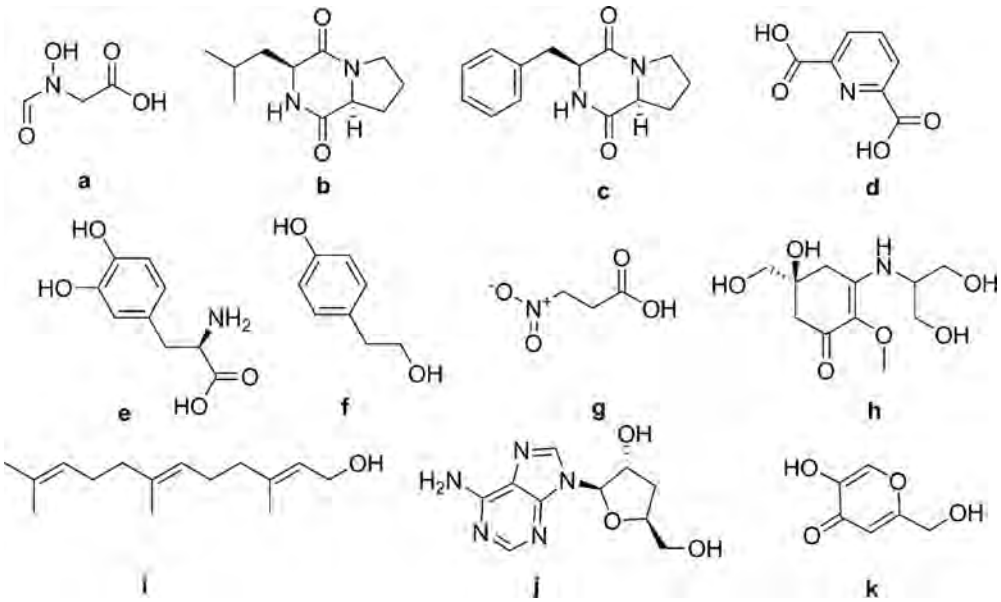


Figure 3 Some rudimentary fungal metabolites. (**a**) Hadicidin. (**b**) Cyclo (L-leucine-L-proline). (**c**) Cyclo (L-proline-L-phenylalanine). (**d**) Dipicolinic acid. (**e**) L-DOPA. (**f**) Tyrosol. (**g**) 3-Nitropropionic acid. (**h**) Mycosporine serinol. (**i**) Farnesol. (**j**) Cordycepin. (**k**) Kojic acid.

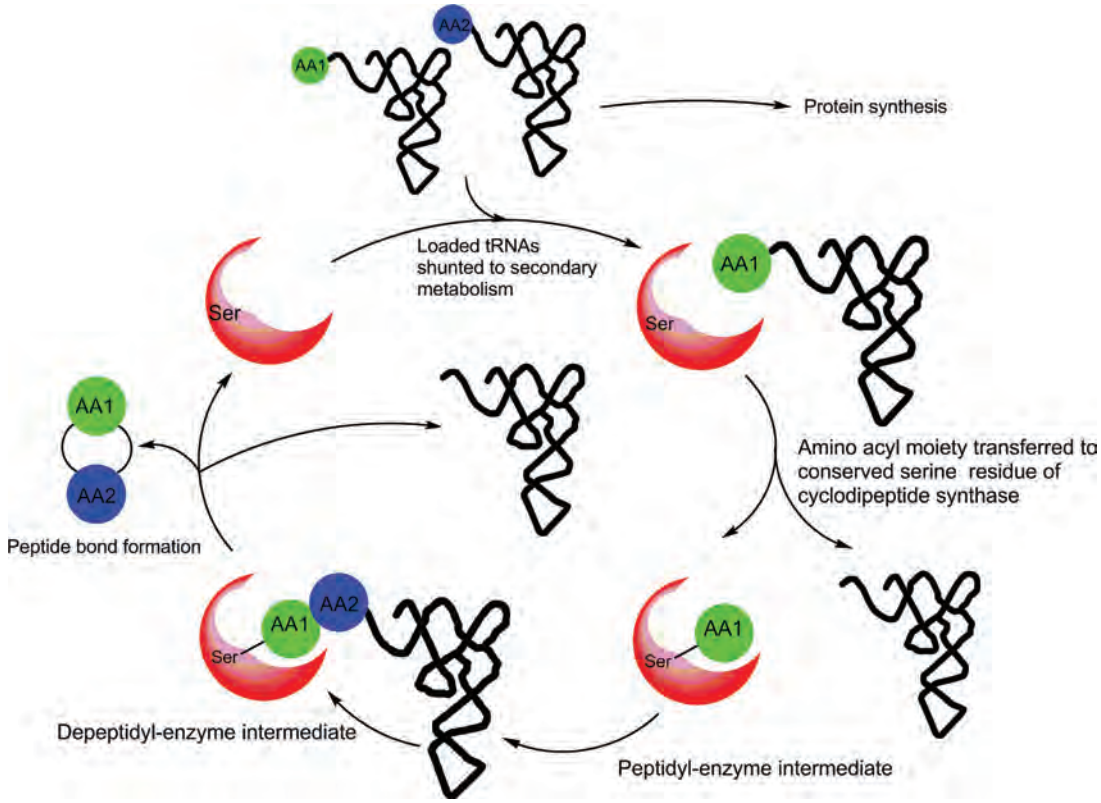


Figure 4 Schematic representation of a cyclodipeptide synthase biosynthetic pathway. A cyclodipeptide synthase (red) binds aa-tRNAs (black) via a serine residue (Ser) to produce cyclodipeptides. aa-tRNAs are generated from an amino acid, ATP, and tRNAs.

intermediate recognizes the aminoacyl-binding site of the cognate aminoacyl tRNA and reacts with the aminoacyl moiety of the second aminoacyl tRNA, forming a dipeptidyl enzyme. This novel enzyme family represents an interesting intermediate between amino acid tRNA-synthetase-based protein synthesis and other nonribosomal processes for peptide bond formation (Fig. 4). Genome mining indicated the presence of CDPS genes in more than 50 bacteria and in at least four *Fusarium* species (104), indicating the presence of a novel mode of fungal peptide synthesis in these fungi.

Many CDPs display biological activities such as inhibition of growth of fungi, bacteria, and human tumor cells. For example, a single fermentation of *A. fumigatus* can produce at least seven CDPs—cyclo (L-Leu-L-Pro) (Fig. 3b), cyclo(L-Phe-L-Pro) (Fig. 3c), cyclo(Gly-L-Pro), cyclo(L-Pro-L-Pro), cyclo(L-Pro-L-Val), cyclo(L-Leu-L-trans-4-OH-Pro), and cyclo(L-Phe-L-trans-4-OH-Pro)—most of which had antibacterial activity. Cyclo(L-Phe-L-Pro), a phytotoxin from *Rosellinia necatrix* (107), attenuated the development of physical dependence on morphine (108). CDPs also provide structural cores for a number of complex fungal secondary metabolites that incorporate diketopiperazine units, including chaetocin, gliotoxin, verticillin B, and many more.

Another simple amino-acid-derived metabolite is dipicolinic acid (pyridine-2,6-dicarboxylic acid) (Fig. 3d), a by-product of the lysine biosynthetic pathway. The synthesis of dipicolinic acid in fungi is thought to be similar to that in bacteria and may occur by condensation of aspartyl-β-semialdehyde with pyruvate to form 2-keto-6-aminopimelic acid, followed by cyclization to form 3,5-dihydro-4-hydroxyl-dipicolinc acid, a hypothetical precursor to dipicolinic acid (109, 110). It is a major component of bacterial endospores, and it is also found in *Penicillium* spp. and various entomopathogenic fungi of the Hypocreales (111). Its role as an extracellular metabolite in *Penicillium* spp. is thought to involve scavenging of external metal ligands, e.g., Mg^{2+}, while in hypocrealean fungi, it may act as an insect toxin (111).

The nonproteinogenic amino acid L-3,4-dihydroxyphenylalanine (L-DOPA) (Fig. 3e) can function as a

secondary metabolite and a virulence factor in fungi. *Cryptococcus neoformans* causes invasive infections in patients with immune systems impaired by AIDS, cancer chemotherapy, or immunosuppressive drug treatments. The fungus produces melanin by transforming shikimic acid to aromatic amino acids, resulting in the formation of L-DOPA. Melanization occurs during growth in its natural habitat in bird excrement and during host infection by the action of a laccase on a variety of precursors, including L-DOPA, D-DOPA, dopamine, and norepinephrine (112). Disruption of genes essential for melanin production in *C. neoformans* caused a reduction in fungal dissemination and lethality in murine infection models (113, 114). Tyrosol (Fig. 3f) is another amino acid-derived metabolite produced by *Candida albicans*, presumably through the decarboxylation of tyrosine; it acts as a quorum-sensing molecule by stimulating the yeast-to-hypha conversion (115).

3-Nitropropanoic acid (3-NPA) (Fig. 3g) has been widely found in some leguminous plants (116) and in some fungi, including *Penicillium* (117) and *Aspergillus* (118) spp. and as a necrotrophic toxin from *Septoria cirsii* (119). Consumption of moldy sugar cane contaminated with 3-NPA produced by *Arthrinium* spp. has led to human deaths in China (120, 121). Its cell toxicity is caused by irreversible inhibition of mitochondrial succinate dehydrogenase (122). Current proposals regarding its biosynthesis are based on the incorporation of ^{14}C-, ^{15}N-, and ^{18}O-labeled substrates into 3-NPA and its derivatives in cultures of *Penicillium atrovenetum*. These experiments showed that ^{14}C is incorporated when the fungus is fed L-[4-^{14}C]aspartate and that an ammonium ion rather than nitrate is involved in forming the nitro group of 3-NPA. The process possibly occurs through a series of oxidations resulting in conversion of the amino group of aspartate to a nitro group with concomitant loss of the aspartate C1 as CO_2. *Phomopsis* species and other endophytic strains excrete high levels of 3-NPA in their culture media, thus raising questions about whether 3-NPA accumulation detected in living plant tissues could be of fungal origin (123).

To resist high-intensity ultraviolet (UV) radiation, cyanobacteria and fungi have evolved biosynthetic pathways to produce hybrid amino acid-containing metabolites called mycosporines to protect vital macromolecules from degradation (124, 125). Mycosporine-like amino acids, e.g., mycosporine serinol (Fig. 3h), were first identified in fungi because of their role in mediating UV-induced sporulation (126). Mycosporine-like amino acids are small (<400 molecular weight), colorless, water-soluble compounds composed of a cyclohexenone or cyclohexenimine chromophore that is derived from a pentose phosphate pathway intermediate, e.g., sedoheptulose 7-phosphate, conjugated with the amino group of an amino acid or its imino alcohol. They are widespread in both the Ascomycota and Basidiomycota (127). Along with melanins, mycosporines absorb UV light, providing photo-protection for the organism. They may also play roles in osmotic regulation (11) and defense against oxidative stress (12). Recently, the biosynthetic pathways for mycosporine and mycosporine-like amino acid formation have been elucidated in cyanobacteria (128) and Actinomycetes (11). Genomic analysis revealed that orthologous biosynthetic potential exists in fungi, suggesting that fungal mycosporines are likely to be encoded by similar gene clusters (128).

Quorum sensing is a means of cooperative intercellular communication via accumulation of small messenger molecules in the environment. As the number of cells increases, the production of messenger molecules increases, and when messenger concentration reaches a threshold, the behavior of the population changes. Quorum sensing was first discovered in various bacteria, but more recently it has been recognized in fungi and has been shown to be relevant for the control of virulence factor expression in *C. albicans*. Supernatants from 2-day cultures of *C. albicans* inhibit the switch from yeast to hyphal growth in new cultures (129). Farnesol (Fig. 3i), a linear sesquiterpene alcohol derived from mevalonate, is the causative agent. *C. albicans* continuously releases farnesol into the environment during growth so that its concentration in the medium is roughly correlated with cell density (129). Farnesol is thought to be secreted by the cells to inhibit germ tube formation in the late stage of biofilm development where there is a high density of interwoven filamentous cells, and therefore, it promotes the dispersal of yeast cells to colonize new environments. Not only is morphological switching essential to *C. albicans* pathogenesis, but farnesol can act as a virulence factor of *C. albicans* by negatively affecting innate immune cells to promote inflammation and a nonprotective and dysfunctional response that fails to activate T cells and produce Th1-promoting cytokines (130). Farnesol can modulate the activity of a transcriptional regulator in *Pseudomonas aeruginosa* when applied as a pure compound or when produced during *C. albicans* coculture, resulting in decreased production of the secondary metabolites pyocyanin and *Pseudomonas* quinolone signal (2-heptyl-3-hydroxy-4-quinolone) while diminishing swarming (131, 132). Farnesol has also recently been implicated in mediating the yeast-hyphal transition

in *Ophiostoma piceae* (133). Additionally, farnesol can induce apoptosis in *Aspergillus* and mammalian cells and inhibits conidial germination and growth in a broad array of fungi and bacteria. Although farnesol is derived from farnesyl pyrophosphate, a central metabolite in steroid and terpene metabolism, the enzyme responsible for hydrolysis to farnesol remains uncharacterized. However, enzyme extracts from *C. albicans* can catalyze formation of farnesol (134).

Nucleoside analogues further exemplify modified primary metabolites that function like secondary metabolites. Cordycepin (3′-deoxyadenosine) (Fig. 3j) is a naturally occurring toxic adenosine analogue and one of the bioactive constituents isolated from *Cordyceps militaris* (135) and *Ophiocordyceps sinensis* (136). Cordycepin enters the cell and is converted to 3′-dATP, which functions as both an RNA chain terminator and a competitive inhibitor of polyadenylate polymerase via interference with subunits of the cleavage and polyadenylation complex (137, 138). A putative biosynthetic pathway for cordycepin was recently proposed based on transcriptome analysis of *O. sinensis*. The analysis identified a putative adenosine kinase, an adenylate kinase, and a 5′-nucleotidase that mediate phosphorylation and dephosphorylation in the process, followed by reduction of adenosine diphosphate to 3′-dADP by a putative ribonucleotide reductase and dephosphorylation to cordycepin (139).

Kojic acid (Fig. 3k) was one of the earliest fungal metabolites to be characterized. It was first isolated from solid-state koji cultures of *Aspergillus oryzae* on rice (140). Kojic acid is a weak, broad-spectrum antibacterial (141), antioxidant, and tyrosinase inhibitor, but its role in the fungal life cycle remains obscure. Early isotope labeling studies suggested that glucose was converted directly to kojic acid by only two or three enzymes and that polyketide or amino acid pathways were not involved (142–145). Recently, its biosynthetic genes were identified by statistical analysis of comparative gene expression profiles (146). Biosynthesis was associated with a small coregulated gene cluster consisting of an oxidoreductase (*kojA*), a transporter (*kojT*), and a transcription factor (*kojR*).

STRUCTURAL CLASSES OF SECONDARY METABOLITES

Shikimate-Derived Metabolites

Shikimate is the deprotonated form of shikimic acid—a trihydroxy cyclohexenecarboxylic acid that provides the carbon skeletons for the aromatic amino acids L-tryptophan, L-phenylalanine, and L-tyrosine via a multistep process leading through chorismate (the shikimate pathway) (147). Thus, any metabolites that incorporate aromatic amino acids in their construction are effectively shikimate-derived. The shikimate pathway is well studied because these essential aromatic amino acids are not produced by mammals and thus constitute important dietary requirements (148). This ancient eukaryotic pathway is highly conserved, because it is found in fungi, bacteria, and plants, and is to a substantial degree an important process involved in primary amino acid metabolism. However, the pathway has undergone diverse evolutionary processes leading to branches that produce a considerable array of secondary metabolites as well, many of which are aromatics and quinones. As noted above, shikimate is also involved in the formation of melanins, as well as mycosporine and mycosporine-like amino acids. Chorismate and subsequent products formed via the shikimate pathway are precursors for the biosynthesis of important isoprenoid quinones such as ubiquinones and menaquinone, which serve important roles in electron transport and as antioxidants. Secondary metabolic products of the pathway are particularly common in plants (flavonoids, coumarins, phenylpropanoids). Fungi tend to display somewhat different kinds of preferences among their shikimate-derived secondary metabolic products (149), because the enzymatic process in fungi is somewhat different from that in plants and bacteria. For example, in fungi, a single complex (AroM) performs five consecutive reactions of the seven basic steps in the pathway, converting 3-deoxy-D-arabino-heptulosonate-7-phosphate (initially formed from phosphoenol pyruvate and D-erythrose-4-phosphate) to 5-enol pyruvyl-shikimate 3-phosphate, which is converted to chorismate in the final step (150).

Simple aromatics often encountered among the fungi, such as benzoic acid derivatives, are commonly derived from this pathway and could be considered as further examples of rudimentary secondary metabolites. *Para*- and *ortho*-hydroxylation patterns tend to occur among shikimate-derived aromatics, in contrast with the *meta*-hydroxylation patterns typical of phenolics arising via the polyketide pathway, and can help in deducing the likely biosynthetic origin of an aromatic compound or unit. More complex shikimate-derived metabolites have been implicated in a variety of roles. For example, involutin (Fig. 5a), a diarylcyclopentenone secreted by the ectomycorrhizal fungus *Paxillus involutus*, reportedly plays a key role as an Fe^{3+} reductant in Fenton-based decomposition of organic matter by mobilizing embedded nutrients,

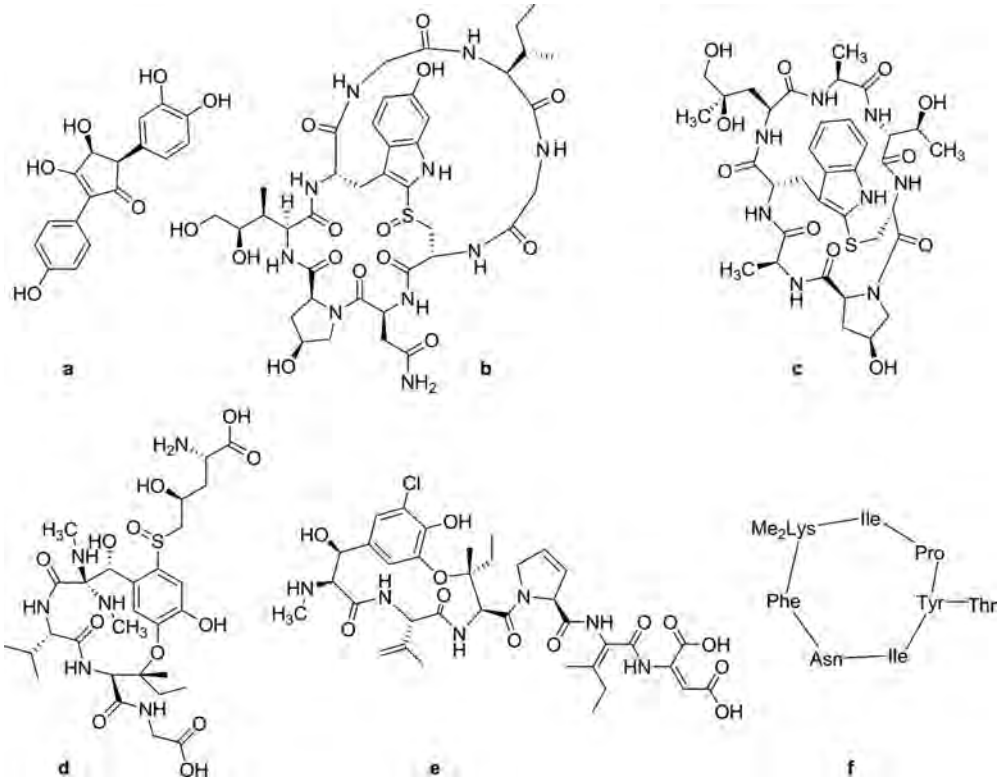


Figure 5 Some fungal metabolites derived from the shikimic acid pathway and ribosomally synthesized and posttranslationally modified peptides (RiPPs). (**a**) Involutin. (**b**) α-Amanitin. (**c**) Phalloidin. (**d**) Ustiloxin A. (**e**) Phomopsin A. (**f**) Epichloëcyclin A.

thereby increasing their accessibility to the host plant (151, 152). Structurally related metabolites, including aryl benzyl lactones such as pulvinic and vulpinic acids, and diaryl quinones, such as polyporic acid, are often encountered as lichen or basidiomycete metabolites (42, 153). *A. terreus* builds related products from *p*-hydroxyphenyl pyruvic acid, e.g. butyrolactones and aspulvinones, from indole pyruvic acid, e.g., asterriquinones (154), and from phenyl pyruvic acid, e.g., phenguignardic acid (155). These types of compounds serve as pigments and antioxidants and can show other bioactivities. Compounds of mixed biogenetic origin that incorporate simple aryl substituents such as phenyl groups (e.g., the antifungal strobilurins, Table 1) are also commonly constructed in part from shikimate-derived precursors.

RiPPs: Ribosomally Synthesized and Posttranslationally Modified Peptides

The influx of sequenced fungal genomes during the past decade has revealed that certain fungal peptide toxins are encoded directly in precursor proteins that are ribosomally synthesized and posttranslationally modified to create toxic peptides (RiPPs). RiPPs are produced in the *Eukaryota*, *Bacteria*, and *Archaea*. Their biosynthetic genes are ubiquitous in genomes, and their products exhibit a vast structural diversity (106, 156, 157). Many RiPPs have cyclic structures that confer the advantage of evading degradation by exopeptidases while increasing structural rigidity that enhances accuracy for binding to their molecular receptors, leading to high binding affinity and biological activity. Furthermore, cyclic RiPPs can often resist denaturation upon heating; thus, their activity is often unaffected by cooking.

The amatoxins and phallotoxins are infamous mushroom toxins produced by various species in the genera *Amanita*, *Galerina*, *Lepiota*, and *Conocybe* (Agaricomycotina) (158–160). They consist of N-to-C cyclized peptides of eight and seven residues, respectively, that also contain several other posttranslational modifications including an unusual 2′-indole thioether bond between the Cys and Trp residues, resulting in a unit referred to as tryptathionine that forms an inner ring structure. The genes (*AMA1* and *PHA1* in *Amanita bisporigera*) encode proproteins of 35 and 34 amino

acids, respectively, in which the amino acid sequences found in the mature toxins are flanked by conserved amino acid sequences. The proproteins are processed by proteolytic cleavage to produce linear peptides comprising the amino acids found in the mature toxins (161). The precursor peptides contain a 10-residue leader peptide, an 8- or 7-residue core peptide for amatoxins and phallotoxins, respectively, and a C-terminal recognition sequence. The tryptathionine in the amatoxin α-amanitin (Fig. 5b) is oxidized to the sulfoxide and hydroxylated on the indole. Other subunits displaying posttranslational modifications include a bishydroxylated isoleucine and *cis*-4-hydroxy proline. Despite their similar structures, the biological activities of amatoxins and phallotoxins are surprisingly different. Amatoxins are potent inhibitors of RNA polymerase II. Phalloidin and phallacidin bind to F-actin (162); thus, they have been developed into commercial kits in which they are conjugated with fluorescent dyes to visualize the cytoskeleton.

RiPPs are well known in other organisms, but several recent studies point to their widespread occurrence in the Ascomycota. Ustiloxins (e.g., ustiloxin A; Fig. 5d) are toxic cyclic peptides from rice false smut, a rice disease caused by *Villosiclava* (=*Ustilaginoidea*) *virens* (163), and also have been identified in *Aspergillus flavus* strains cultured on maize (164). They are generally constructed of a Tyr-Val/Ala-Ile-Gly tetrapeptide sequence and cyclized via an ether linkage of the side-chains of modified tyrosine and isoleucine units. It was long thought that ustiloxins A and B were biosynthesized in *V. virens* by a nonribosomal peptide synthetase (165). Analysis of coregulated transcripts in *A. flavus* identified a 15-gene cluster. The cluster's core gene, *ustA*, encoded UstA, a peptide with 16 repeats of a Tyr-Ala-Ile-Gly tetrapeptide unit that is converted into the cyclic moiety of ustiloxin B (164, 166). This highly repetitive sequence for the core peptide indicates that the *ustA* gene might have undergone selection for increased production of ustiloxin B. Adjacent to *ustA* in the gene cluster are gene(s) that encode a peptidase (*ustP1* and *ustP2* as a putative single gene), indicating that a peptide sequence served as the enzyme's substrate. Norvaline is then transferred to the activated aromatic ring of the ustiloxin tyrosine residue by a glutathione S-transferase (UstS). Sequence similarity searches with the ustiloxin biosynthetic gene cluster from *A. flavus* were used to probe the genome of *V. virens* (167) to identify a 15-gene cluster containing a gene encoding a precursor protein with five Tyr-Val-Ile-Gly and three Tyr-Ala-Ile-Gly repeats for ustiloxins A and B, respectively.

Phomopsins (e.g., phomopsin A; Fig. 5e) comprise a family of cyclic hexapeptide mycotoxins produced by *Diaporthe leptostromiforme* that infects lupins (*Lupus* spp.) (168). Phomopsins are comprised of a 13-membered macrocyclic ring formed by an ether bridge linking an isoleucine residue to the aromatic ring of a highly modified tyrosine unit. Lupin forage contaminated with phomopsins causes lupinosis, a liver disease of sheep and cattle. Phomopsins are potent microtubule inhibitors, and their toxicity is caused by their binding of the phomopsin tripeptide side chain to the vinca domain of tubulin, resulting in potent antimitotic activity (169). The ustiloxins contain a similar modified-tyrosine-containing macrocycle. The putative precursor peptide sequence (YVIPID) was used to search the genome of *D. leptostromiforme*, resulting in the identification of a precursor peptide gene, *phomA*, whose product contained five repeats of short peptides with the sequence YVIPID. The Phom gene cluster also included a copper-containing tyrosinase (PhomQ), an S41 family peptidase (PhomP1), an *S*-adenosylmethionine-dependent methyltransferase (PhomM), and other functionally unknown proteins (170). Disruption of PhomQ, the tyrosinase responsible for formation of the cyclic scaffold of phomopsins, abolished phomopsin biosynthesis.

A combination of two functionally unknown genes (*ustYa* and *ustYb*) and the *ust B* precursor gene were used to search genomes of other *Aspergillus* spp. At least 94 sets of precursor candidates were computationally identified and classified, indicating that RiPPs are likely to be widespread in the Ascomycota. Furthermore, one of these newly identified candidate RiPPs with two types of repeated sequences was identified by gene inactivation in *A. flavus* and found to be a novel bicyclic peptide that was named asperipin-2a (171). Similarity searches with the PhomA, PhomQ, and PhomM homologous proteins identified an additional 27 gene clusters similar to that involved in phomopsin biosynthesis in a variety of ascomycetes (170). Furthermore, Phom-like gene clusters were recognized in strains of *Beauveria bassiana* and *Metarrhizium aniosipliae*, leading to the fermentation, purification, and characterization of new phomopsin A analogs from *B. bassiana* (phomopsin Z) and *Metarhizium anisopliae* (metarisin A and B).

One of the most highly expressed *in planta* fungal transcripts in *Epichloë* endophyte-infected grasses, designated *gigA* (for grass-induced gene), was identified from tall fescue and perennial ryegrass associations (172). The protein sequence encoded by *gigA* was predicted to be a variable 27-amino acid repeat with an

N-terminal signal sequence, indicating that it should be translocated into the Golgi apparatus. The region adjacent to *gigA* contained a kexin protease gene for processing GigA for the liberation of multiple cyclic nonapeptides, the epichloëcyclins, into the host plant. The structures of epichloëcyclins A to E (e.g., epichloëcyclin A; Fig. 5f) were determined in part by the sequences of individual repeat units within the GigA protein, which differed between fungal strains and within the proprotein itself. Such processing of a single protein into multiple different small peptides is a remarkable and novel mode for generating chemical diversity. Gene deletion of *gigA* led to the elimination of all epichloëcyclins, with no conspicuous phenotypic impact on the host grass (172). The *gigA* gene cluster has also been identified in other plant-symbiotic Clavicipitaceae including *Periglandula ipomoeae* and *Atkinsonella hypoxylon*, suggesting a possible widespread bioactive role in plant-fungus symbioses.

Polyketides

Polyketides are the largest and most structurally diverse group of fungal secondary metabolites. Their carbon scaffolds share a common biosynthetic origin and are derived from the polymerization of short-chain carboxylic acid units (e.g., acetate, malonate). Polyketides are biosynthesized by large multifunctional enzymes called polyketide synthases (PKSs) and can be classified into four main types according to the catalytic domains and enzymatic mechanisms involved in their formation (173). Fungal PKS enzymes are closely related to fatty acid synthases. In the Ascomycota and Basidiomycota, the prevalent types of PKSs belong to the iterative type I class in which the domains act repetitively on the growing polymer during each chain extension cycle. The carbon backbones are assembled by repetitive decarboxylative Claisen condensation catalyzed by β-ketoacylsynthase domains and employ malonyl thioesters as extender units. An acyl carrier protein domain acts as an anchor where the growing polyketide chain is covalently tethered. After each chain-extension step, the polyketide backbone is increased by one "ketide" unit. Additional domains including β-ketoreductases, dehydratases, and enoylreductases can selectively modify the newly formed polyketide. Unlike fatty acid synthases, in which most or all of the β-keto positions are completely reduced to methylenes, resulting in a fatty acyl product, the tailoring domains in PKSs act in preprogrammed patterns at each β-position, resulting in products of varied oxidation states. Furthermore, one or more methyl groups may be introduced at the α-position of the growing chain by C-methyltransferase domains. Finally, the fully extended chain is usually offloaded or cyclized by a thioesterase domain, or in other cases, a terminal acyl carrier protein domain transfers the tethered product to an acyl transferase or other protein that shuttles it and covalently links it with another biosynthetic product. Thus, the collection of domains functions in an iterative fashion and follows integral programming rules that determine chain length, cyclization, reductive tailoring, and other structural features (174, 175).

Fungal iterative PKSs can be further grouped into three major subtypes: nonreducing (or aromatic) (NR-PKS), partial reducing (PR-PKS), and highly reducing (HR-PKS) PKSs, depending on the degree of programming and extent of α- and β-carbon processing. NR-PKSs are among the best-understood types of fungal PKSs. Griseofulvin (Fig. 6a, Table 1) was first characterized from *Penicillium griseofulvum* in 1939 and later was recognized for its antifungal effects, resulting in it being named the "curling factor" because it induced curling of fungal hyphae (176). Griseofulvin was developed at Glaxo Laboratories for the treatment of ringworm and other infections by dermatophytes. Genome scanning and gene deletion studies established that an NR-PKS, *gsfA*, encodes biosynthesis of the griseofulvin heptaketide backbone and that gsfI is the halogenase involved in chlorination of the final product (177, 178). Far fewer PR-PKSs have been characterized, with the 6-MSA (Fig. 6b) PR-PKS being a biosynthetically pivotal and widespread gene. The 6-MSA PR-PKS from *Penicillium patulum* was the first PKS to be purified and sequenced, and 6-MSA is an intermediate in the biosynthesis of the mycotoxin patulin (179). The *atX* gene from *A. terreus* and the *PKS2* gene from *Glarea lozoyensis* also encode for the 6-MSA PKS (180, 181). A fungal PR-PKS is responsible for the synthesis of the widespread metabolite (*R*)-mellein (Fig. 6c) in *Parastagonospora nodorum* (182) and probably many other fungi. It was proposed that biosynthesis of (*R*)-mellein was highly parallel to that of 6-MSA, requiring an additional chain elongation and keto reduction. These PR-PKS genes are likely important in encoding the biosynthesis of other dihydroisocoumarins and possibly the mycotoxin ochratoxin (183). Phylogenetic analyses have shown that the 6-MSAs and other uncharacterized PR-PKSs are more distantly related to the NR- and HR-PKSs and more closely resemble bacterial modular type I PKSs, suggesting a common origin from bacteria (184). However, divergence between the 6-MSA- and mellein-type PR-PKSs likely occurred during the course of fungal radiation (182).

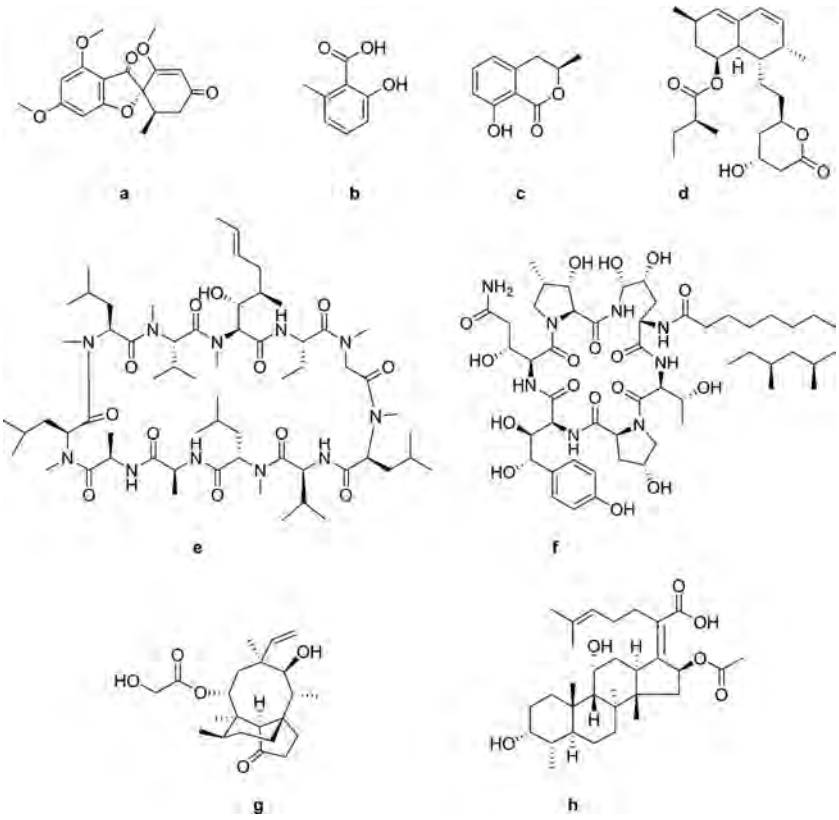


Figure 6 Some fungal polyketides, nonribosomal peptides, and terpenoids. (**a**) Griseofulvin. (**b**) 6-Methyl salicylic acid. (**c**) (R)-Mellein. (**d**) Lovastatin. (**e**) Cyclosporine A. (**f**) Pneumocandin A_0. (**g**) Pleuromutilin. (**h**) Fusidic acid.

The HR-PKSs have a higher degree of complexity in their programming relative to that of the NR-PKSs. T-toxin, the main virulence factor involved in southern corn leaf blight, a devastating disease caused by *Drechslera heterostrophus* race T that attacks maize, is a typical HR-PK. It consists of a linear polyketide whose biosynthesis is encoded by a nine-gene cluster that includes two cooperating HR-PKSs (185, 186). Fumonisins comprise a major group of mycotoxins produced by *Fusarium* spp. and certain black *Aspergillus* spp. (187). They act as ceramide synthase inhibitors. Coexpression of 15 contiguous genes defines a fumonisin biosynthetic gene cluster in *Fusarium moniliforme*, of which FUM1 is an HR-PKS that assembles the fumonisin backbone (19).

The compactin and lovastatin biosynthetic pathways are among the best-studied examples of HR-PKSs. These metabolites were among the first natural product enzyme inhibitors discovered for use in human health. As early as the mid-1960s, the biochemical pathway of cholesterol biosynthesis was fairly well understood, and the enzyme hydroxymethylglutaryl-CoA reductase (HMGR) was known to convert HMG-CoA to the intermediate mevalonate. Akira Endo at Sankyo in Japan developed a three-step assay (two phenotypic screens and a target-based assay) using liver tissues extracted from rats to screen fermentation broths for natural products that specifically inhibited HMGR (188). Using this approach, Endo discovered the compound ML-236B, later named compactin (Table 1), from a strain of *P. citrinum* (189). Although compactin was not developed as a drug, Endo and a group at Merck in the United States independently used strategies analogous to that used to find compactin to discover lovastatin, a methylated analog of compactin, from two fungi, *Monascus ruber* and *A. terreus*, respectively (188). Merck developed lovastatin (Fig. 6d), which became the first-in-class member of the statins (190), compounds that are widely and successfully used to treat hypercholesteremia. While this natural product was highly effective, a slight structural modification led to Zocor (simvastatin), a blockbuster drug product peaking at over $5 billion/year in sales during its patent lifetime. Using these compounds as models, chemists at

Warner-Lambert designed the chemically distinctive compound atorvastatin (Lipitor), which became the best-selling drug of all time, with more than $125 billion in sales over its patent lifetime. These and other second-generation synthetic derivatives or analogues of compactin and lovastatin continue to be among the world's best-selling drugs.

Closely related to the products of HR-PKSs are a broad range of hybrid polyketide-amino acid metabolites formed by fusion of an HR-PK to an amino acid-binding domain and known as iterative PKS-NRPSs (174, 191). Functionally characterized fungal iterative PKS-NRPS hybrid gene clusters have been linked to the biosynthesis of tetramic acid-containing metabolites such as equisetin, the actin-binding cytochalasins, the immunosuppressive agent pseurotin A, the nematocidal thermolides, and the mycotoxins fusarin C and cyclopiazonic acid, among others.

NRPs: Nonribosomal Peptides

The Fungi offer one of the richest and most highly elaborated pools of NRPs among all organism types. This diversity reaches its zenith in the filamentous Ascomycota (192, 193). The corresponding NRPS megaenzymes synthesize a wide range of biologically active natural peptide compounds. NRPS-derived siderophores are involved in iron transport, storage and homeostasis, and virulence toward plants and animals (194–196). NRPSs are responsible for the biosynthesis of the iconic natural product-based drugs penicillins, cephalosporins, cyclosporine A, and echinocandins (Table 1). NRPSs are similar to PKSs in their modular structure and share an assembly line thiotemplate mechanism to achieve peptide synthesis (197). Like PKSs, minimal NRPS modules have an analogous set of three core functional domains: adenylation (A) domains that recognize and activate a specific amino acid or amino acid derivative by adenylation with ATP, peptidyl carrier protein domains (also known as thiolation [T] domains) that bind the activated substrate to a 4′-phosphopantetheine with a thioester bond and shuttle the substrate, and condensation (C) domains that catalyze peptide bond formation between adjacent monomer units on the NRPS complex.

The selection of a highly diverse set of monomers for peptide synthesis, including a plethora of nonproteinogenic amino acids, contributes to the overall complexity of NRPs. Although exceptions occur, generally, fungal multimodular NRPSs follow the collinearity rule, in which a complete A-C-T module corresponds to one amino acid in the NRP amino acid sequence. Further structural diversification accrues during chain initiation, chain elongation, and chain termination of NRP biosynthesis. Some NRPSs, e.g., echinocandins, carry an N-terminal T and C domain (or starter C domain) before the first NRPS module to prime the synthesis of the peptide portion by addition of an aliphatic acyl chain. NRPSs may incorporate other catalytic domains which modify the substrate during biosynthesis, including (i) an epimerization (E) domain that inverts the chirality of the last amino acid in the growing peptide from the L- to the D-configuration, (ii) an *N*-methylation (M) domain (methyltransferase) that transfers a methyl group from an *S*-adenosylmethionine unit to the α-amino group of an amino acid substrate, and (iii) a specialized domain termed a cyclization (C) domain which catalyzes internal cyclization to oxazoline or thiazoline rings constructed from cysteine, serine, or threonine units. Often, additional tailoring enzymes may lie adjacent to the core NRPS and modify either the monomers or the final peptide product by acylation, glycosylation, hydroxylation, or halogenation. Offloading and release by macrocyclization is a common modality in fungal NRPSs and constrains the peptide backbone in a biologically active conformation. Fungal NRPSs characterized thus far universally terminate macrocyclic peptide synthesis with a condensation-like C_T domain that catalyzes the cyclization reaction.

The rapid development of therapeutic methods and industrial-scale processes for production of the β-lactam antibiotic penicillin during the 1940s were arguably the most significant milestones in the history of drug discovery research (198). Substantial increases in the human life span can be traced back to the introduction of the β-lactam antibiotics penicillin and cephalosporin and their modern derivatives (Table 1). The penicillin and cephalosporin biosynthetic gene clusters are among the best characterized of all NRPSs (199). Recently, the penicillin gene cluster from *Penicillium chrysogenum* was reconstituted as a single synthetic three-open reading frame polycistronic gene, and the construct was placed under the control of a single promoter (200). The open reading frames were separated by a sequence encoding the viral 2A peptide, which resulted in cotranslational cleavage, hence resulting in the formation of independent enzymes. After yeast-mediated homologous recombination, random genomic integration of this construct resulted in penicillin production in the heterologous host *A. nidulans* (200).

The introduction of the undecapeptide immunosuppressant drug cyclosporine A (Fig. 6e, Table 1) transformed and enabled the field of organ and tissue transplant methods. The genomic sequence of *Toly-*

pocladium inflatum and RNA-Seq transcriptome analysis has recently defined the complete gene cluster for cyclosporine biosynthesis (201). The formidable cyclosporine NRPS SimA, with its 11 amino acid-activating modules, seems to have evolved via duplication of simpler NRPSs that are apparently shared among other fungi of the Hypocreales. Several other NRPSs responsible for the biosynthesis of smaller cyclic depsipeptides in Hypocrealean fungi, such as enniatin, beauvericin, and bassianolide synthetases, and in part destruxin synthetase, appear to form a monophyletic lineage within the SimA clade (201). The biosynthetic pathways for the pneumocandins (e.g., Fig. 6f) in *G. lozoyensis* (202, 203) and echinocandin B in *Aspergillus pachycristatus* (204, 205), the starting metabolites for the semisynthesis of the lipopeptide antifungal drugs Cancidas and Eraxis, respectively (Table 1), have been mapped at the genetic level and functionally characterized to some extent. Phylogenetic studies from pathway gene clusters from multiple echinocandin-producing fungi have demonstrated that the echinocandin gene clusters have a common ancestor and formed a unique lineage among the families of fungal NRPSs (206).

Terpenoids

Fungi of the Ascomycota and Basidiomycota are prolific producers of terpenoids. In the Basidiomycota, terpenoids, especially diterpenoids and sesquiterpenoids, are the dominant biosynthetic class of secondary metabolites. In-depth information on the spectrum of fungal terpenoids can be found in recent reviews (26, 207, 208). Terpenoids are assembled from five-carbon precursor molecules: isopentenyl diphosphate and/or dimethylallyl diphosphate. In fungi, these two isomers are derived from acetyl-CoA via the mevalonate pathway (209). Condensation of isopentenyl diphosphate and dimethylallyl diphosphate monomers yields branched hydrocarbons in multiples of five carbons that are termed as being constructed of isoprene or prenyl units: geranyl pyrophosphate (C_{10}), farnesyl pyrophosphate (C_{15}), geranylgeranyl pyrophosphate (C_{20}), squalene (C_{30}), and phytoene/lycopene (C_{40}). These linear prenyl chains are further processed by prenyl transferases, terpene cyclases, and terpene synthases, resulting in a cascade of dephosphorylation, further condensations, and cyclizations which configure the carbon skeleton of the different classes of terpenoid natural products. Occasional loss of one or more methyl groups during biosynthesis results in norterpenoids. In addition, polyketide-, sugar-, or amino acid-derived elements are sometimes incorporated to yield molecules of mixed biosynthetic origin. For example, a hybrid terpenoid-polyketide is often referred to as a meroterpenoid. We mention only a few representatives from this vast class of fungal metabolites below.

The topical antibacterial antibiotic retapamulin is synthesized from the diterpenoid pleuromutilin (Fig. 6g; Table 1) produced by the mushroom *Clitopilus passeckerianus* and some related species (210, 211). A patent application from Sandoz AG first described the pleuromutilin pathway as consisting of six transcriptionally coregulated genes, including a labdane-type diterpene synthase gene, a putative geranylgeranyl diphosphate synthase gene, three cytochrome P450 monooxygenase genes, and a putative acyltransferase-encoding gene (212). The cDNA sequences of seven genes (*Pl-ggs*, *Pl-cyc*, *Pl-atf*, *Pl-sdr*, *Pl-p450-1*, *Pl-p450-2*, and *Pl-p450-3*) constituting the gene cluster in *C. passeckerianus* were cloned and placed under control of constitutive promoters for expression in *A. oryzae* (213). One of the *A. oryzae* transformants gave a remarkable 20-fold increase in pleuromutilin titers compared to the native host. This outstanding achievement represents the first successful heterologous expression of an entire basidiomycete secondary metabolite gene cluster in an ascomycete host and paves the way for further improvement of the pleuromutilin-producing *A. oryzae* strain through classical mutagenesis and other genetic manipulations (213).

Fusidic acid (Fig. 6h, Table 1) is a fusidane-type triterpenoid produced by *Acremonium fusidioides* that has long been used as an oral antibiotic for treatment of Gram-positive bacterial infections (214). Although labeled isotope studies have established that its precursor is squalene (215), its gene cluster has yet to be characterized. Nevertheless, its biosynthesis is likely to be initiated by an oxidosqualene synthase similar to that found in *A. fumigatus, Metarhizium anisopliae* (216), and *Sarocladium caerulens* (217), all of which produce the closely related helvolic acid. In *A. fumigatus*, the helvolic acid gene cluster has been characterized and comprises a gene for an oxidosqualene cyclase (*AfuOSC3*) that is colocalized with genes of cytochrome P450 monooxygenases, a short-chain dehydrogenase/reductase, and an acyltransferase, which presumably function in triterpene-tailoring steps (218, 219).

The genetic basis of the biosynthesis of trichothecene-type mycotoxins, which share a common tricyclic 12,13-epoxytrichothec-9-ene core structure, has been identified in fusaria and other species of the Hypocreales (220, 221). The trichothecene family also illustrates the incompleteness of our knowledge of the distribution of fungal biosynthetic gene clusters; trichothecolone acetate was recently discovered in the

apple sooty blotch fungus *Microcyclospora tardicrescens* (222). Indole-diterpenoid metabolites have been investigated intensively because of their neurotoxic and other biological effects. They are distributed sporadically among fungi of the Sordariomycetes and Eurotiomycetes. These metabolites consist of a diterpene backbone derived from geranylgeranyl diphosphate and an indole moiety derived from indole-3-glycerol phosphate (223). The biosynthetic gene clusters for paxilline (224, 225), aflatrem (226, 227), the lolitrems (228), penitrem (229), and terpendole E (230) have been functionally characterized. Finally, the heterologous expression of the products of several synthetic ascomycete monoterpene and sesquiterpene synthases has been achieved (231, 232), resulting in *in vitro* production of potentially valuable terpenoids, e.g., eucalyptol, pinenes, guaiene, caryophyllene, chamigrene, and gurjunene, for use as fuel additives, fragrances, and other commodities.

READING THE SECONDARY METABOLITE MENU AND BIOINFORMATICS-BASED DISCOVERY OF NATURAL PRODUCTS

Knowledge of microbial secondary metabolites is highly advanced due to the resources invested in controlling microbial toxins, crop virulence factors, and discovery of their chemical applications in medicine and agriculture. Studies of natural products produced by fungi have provided powerful probes for cell biology and drugs useful in medicine. Their importance is evidenced by their prominence as reagents and metabolic blockers for cellular metabolism and for labeling cell macromolecular structures. Understanding the biosynthetic logic of the principal pathways and how they operate can often enable straightforward recognition of the biogenetic origin of a given secondary metabolite. The metabolic precursors of metabolites can also be identified by feeding strains isotopically labeled precursors. Previous work in this area has served as the foundation for modern genetic biosynthetic studies (27, 233). Computational methods for decoding secondary metabolite pathways from sequenced genomes have transformed natural products research and have begun unfolding the roadmap to secondary metabolite biosynthetic genes and their regulation. Rapid advances in technology for genome sequencing and analysis are accompanied by parallel advances in relevant aspects of analytical chemistry. Applications of new techniques and informatics tools to mass spectrometric analysis are effectively expanding the field of metabolomics to encompass secondary metabolites (234, 235). These techniques are also fostering efforts to explore the significance of secondary metabolites, e.g., by detecting and localizing them *in situ* during interspecies interactions (236). Similarly, nuclear magnetic resonance capabilities continue to improve in terms of sensitivity, speed, pulse sequence development, automated interpretation, and amenability to mixture analysis (237). For example, the gene clusters and essential biosynthetic steps have been characterized for most major mycotoxins and fungus-derived drugs. The tools are sufficiently accessible that discoveries of important new microbial metabolites are now accompanied by characterization of the corresponding encoding gene clusters (238, 239). Within the next few years, we expect to see a near complete catalogue of gene clusters of most of the important fungal secondary metabolites (32).

Until recently, discovery of fungal metabolites was largely an empirical process that was supported by ecological, phylogenetic, and physiological clues to what fungal strains were most likely to produce secondary metabolites and by knowledge of which laboratory conditions would most likely produce metabolite-rich fermentations. The universe of known fungal metabolites has been further expanded from studies of fungi of the Basidiomycota and Ascomycota that produced large or numerous fruiting structures that could be collected in the field and then extracted directly for chemical studies. Metabolites characterized directly from fruiting bodies of ectomycorrhizal fungi in many genera, e.g., *Cortinarius*, *Boletus*, *Lactarius*, *Hydnellum*, *Cantharellus*, and those directly extracted from lichenized fungi have provided a vast catalogue of secondary metabolites from species that have proven to be unculturable in the laboratory (42, 240). While the prokaryotic biology community sensationalized their realization of the "uncultured majority" in the 1980s and 1990s (241), fungus biologists have been aware of vast numbers of unculturable fungi since axenic cultures were introduced in the 1800s (242) and have generally assumed that unculturability was a normal phenomenon. Thus, issues of unculturability of fungi were acknowledged early on in the history of fungal chemical research and differ significantly from those associated with prokaryotic organisms. Unlike most prokaryotes, fungi can often be seen by eye or by low magnification microscopy, and their cells and tissues harvested for their DNA and chemistry. Consequently, the issue of unculturability has had less impact on efforts to evaluate biodiversity and chemical diversity in fungi, even though it remains a significant hurdle in the experimental study of biosynthesis and for practical scale-up of metabolite production.

Secondary metabolite gene clusters are among the most diverse and rapidly evolving features of fungal genomes and can be used to follow mechanisms of adaptive evolution, including evolution of pathogenic species (44, 243, 244). During the past decade, the search for fungal metabolites, like that for other microorganisms, and their corresponding biosynthetic genes has entered a transformative new era. The search for new and useful microbial metabolites is shifting from being an experience-guided empirical process to being a genomics-guided process in which identification of the biosynthetic genes precedes and informs isolation of the secondary metabolite. By examining historical data retrospectively, it is now possible to rationalize why a strain's genetic makeup dynamically interacts with fermentation methods to yield fluctuating patterns of bioactive molecules during screening and why the pool of new drug-like metabolites from certain kinds of common fungi appeared to have been depleted. The numbers of biosynthetic gene clusters in every filamentous genome examined to date is thought to exceed the number of detectable metabolites under a given set of laboratory conditions, indicating the existence of numerous unexpressed or poorly expressed pathways possibly encoding the biosynthesis of undescribed metabolites. These unexpressed gene clusters are commonly called "orphan" or "cryptic" gene clusters because their silence is likely an artifact caused by our inability to simulate the environmental conditions or chemical signals required for their expression, or even to grow the organism at all (245, 246). Regulation of secondary metabolism remains poorly understood across the diversity of secondary metabolite-producing organisms, and identification of a low-abundance discrete predicted metabolite from within a crude metabolome is a nontrivial challenge. Recognition that transcription and translation of these repressed genes can sometimes be induced has spawned an entirely new area of natural products research dedicated to finding chemical and genetic tools to break pathway silence and induce homologous expression in the native organism (71, 245, 247–249).

Genome sequencing has moved beyond model fungal species, major pathogens, and industrial organisms to encompass the full spectrum of fungal diversity. Consequently, as in other microorganisms, a global picture of the phylogenetic distribution of secondary metabolites encoding genes and gene cluster architecture in fungi is emerging. In principle, conceptual barriers no longer impede the future unlocking of cryptic secondary metabolic gene clusters. The complex secondary metabolism of a given fungus can easily constitute 20 to as many as 80 different core secondary metabolite-encoding genes or gene clusters. On the other hand, fungi that have little history of secondary metabolite production, e.g., chytrids, yeasts, powdery mildews, vesicular-arbuscular mycorrhizae, and truffles, are now known to lack or have few secondary metabolite gene clusters because (i) they diverged prior to the evolution of secondary metabolism or (ii) during their descent from fungal groups with rich secondary metabolism, their genomes underwent a concurrent loss and reduction in unnecessary secondary metabolism due to stable environments provided by obligate parasitism (e.g., rusts, smuts, powdery mildews) (250). However, other biotrophic fungi, e.g., lichenized fungi, mycorrhizal mushrooms, and plant-associated Clavicipitaceae, have retained a rich secondary metabolism that is highly related to that of their metabolite-rich sister phylogenetic lineages. It is hypothesized that these fungi have retained their secondary metabolic capabilities in part because of the need for defense and the interorganism interactions of their conspicuous and often long-lived vegetative (lichens) or reproductive (mushrooms) structures, or to maintain their mutualistic relationships with host plants (Clavicipitaceae) (44, 240, 251–253).

Reading the biosynthetic menu for secondary metabolites from sequenced fungal genomes is a complex process, but it can be assisted by several computational tools that can identify signature motifs and genes associated with secondary metabolite biosynthetic gene clusters or, minimally, the primary catalytic backbone genes (254). A full range of software packages can be found at the Secondary Metabolite Bioinformatics Portal (http://www.secondarymetabolites.org). These software packages organize searches and categorize results so that identified genes and gene clusters are associated with their nearest known orthologues. In some instances, these searches can propose a hypothesis for a secondary metabolic product of a cluster through close homology with gene clusters that have been experimentally linked to a secondary metabolite.

The most widely used software tool is the Antibiotics & Secondary Metabolite Analysis Shell (antiSMASH, http://antismash.secondarymetabolites.org) (255). This Web-based tool enables users to easily identify and annotate pathways with typical repetitive domain structures directly from DNA or protein sequence data. Two complementary tools are designed to aid in organizing the results of AntiSMASH analyses. One is the ClusterMine360 database that stores the antiSMASH annotation of known gene clusters and links them to its own repository or to sequence references in the NCBI GenBank as well as to the structure

of the natural product encoded by the gene cluster (256). The other is the recently released Minimum Information about a Biosynthetic Gene cluster (MIBiG), a Web-based community-annotated database which has cataloged about 200 unique natural product pathways including those corresponding to most classes of fungal metabolite. This effort is vital to guiding future genome mining and synthetic biology efforts aimed toward discovering novel fungal enzymes and metabolites. Other computational tools useful for fungi include Secondary Metabolite Unknown Regions Finder (SMURF, http://jcvi.org/smurf/index.php) (257); Cluster Assignment by Islands of Sites (CASSIS), a method for biosynthetic gene cluster prediction in eukaryotic genomes, and Secondary Metabolites by InterProScan (SMIPS), a tool for genome-wide detection of key secondary metabolite backbone enzymes (anchor genes), e.g., PKSs, NRPSs, and dimethylallyl tryptophan synthases (https://sbi.hki-jena.de/cassis/cassis.php) (258); ClustScan (http://bioserv.pbf.hr/cms/) (259, 260); and natural product searcher (NP.searcher, http://dna.sherman.lsi.umich.edu/) (261).

Currently, for fungal gene clusters, *de novo* structure prediction from gene cluster DNA sequence data may provide a structure hypothesis when the targeted genes exhibit high homology to a functionally characterized gene cluster of a known chemical product. Detailed manual prediction of structure from gene cluster data can often provide a reasonable estimate for the core structure encoded by a PKS or NRPS, but automated structure prediction is not yet possible.

More recently, alternative methods have been developed to identify clusters for which the genes are cooperatively regulated at transcriptional levels, thus allowing for the identification of novel gene clusters even when sets of canonical core genes are unrecognizable (254, 262). The cases of kojic acid and ustiloxin B, mentioned above, demonstrated that different computational techniques can be applied to identify biosynthetic gene clusters that do not contain any known types of core scaffold-synthesizing enzymes. Discovery of the corresponding gene clusters employing the MIPS-CG algorithm has been applied to two or more transcriptional data sets from producing and nonproducing conditions to identify chromosomally adjacent clusters of coexpressed genes over a 3- to 30-gene frame in a fungal genome. The advantage of this approach was that no assumptions were made regarding the patterns of the genes that would be identified which can help recognize hypothetical new classes of biosynthetic gene clusters. The expanding use of these tools has led to the observation that most newly identified biosynthetic pathways are not readily associated with a known PK, NRP, terpenoid, or other product. The assignment of potential products to these orphan pathways is thus a major unaddressed challenge, as is the basic question of whether these newly recognized biosynthetic gene clusters are even functional (254).

A global overview of secondary metabolite gene cluster evolution and diversification in the Fungi is now emerging, and significant progress has been made in developing predictive frameworks for classifying fungal NRPSs, PKSs, and terpene synthases. Previously, the frequency of isolation of bioactive metabolites and the chemical complexity of organic extracts were used to measure the chemical potential of different fungal taxa. The genetic capacity of different groups of fungi can now be explored directly from genomic sequences to ask which taxa have the highest potential to produce large numbers of complex secondary metabolites with drug-like properties and which taxa have moderate or poor potential. Because most drug or drug-like metabolites are polyketides, nonribosomal peptides, or terpenoids or incorporate derivatives of dimethylallyltryptophan, the numbers of iterative PKS, NRPS, terpene synthase, prenyl transferase, and dimethylallyltryptophan synthase genes per genome can serve as a measurement of the potential to produce secondary metabolites. On this basis, the dikaryotic filamentous fungi are clearly the most productive sources of secondary metabolites compared to the basal fungal lineages. This conclusion correlates very well with historical data on how the numbers of known secondary metabolites are distributed across the Fungi.

The highest complexity of secondary metabolite pathways is concentrated within the filamentous Ascomycota, especially in the higher lineages of the Eurotiomycetes, Dothideomycetes, Sordariomycetes, Leotiomycetes, and Lecanoromycetes, while secondary metabolism is limited or absent altogether in the obligate biotrophic Erysiphales, the basal lineages of the Pezizomycetes, the Saccharomycotina, and the Taphrinomycotina (Fig. 1). All genomes of the higher ascomycete lineages examined to date have roughly a minimum of 5 to 10 core secondary metabolic pathways (pigments, siderophores) and may have as many as 80 (263), thus rivaling the complexity and richness of the Actinomycete genus *Streptomyces*. In particular, gene clusters for biosynthesis of polyketides and nonribosomal peptides have undergone significant expansion, and many of these pathways are ascomycete-specific. Furthermore, few of these core biosynthetic genes are fully conserved across ascomycete and basidiomycete fungi; therefore, when one takes into account

the potential numbers of fungal species, the capacity for encoding chemical diversity in the Ascomycota seems limitless. The situation in the Basidiomycota appears to be nearly as complex, but with the chemistry and diversification of gene clusters skewed toward terpenoids. The Agaricomycotina have genomes that are often rich in terpene pathways, are moderately rich in PKSs, and incorporate only a few NRPSs (240, 264–266), while other Basidiomycota lineages have little or no secondary metabolism due to evolutionary contraction as a consequence of biotrophy and parasitism (6, 267, 268).

CONCLUDING REMARKS

There is an urgent a need for the development of new drugs to combat multidrug-resistant pathogens and for molecular templates for targeted cancer therapeutics. The capacity for sequencing microbial genomes is outpacing the ability to identify natural product gene clusters and, most importantly, to convert this sequence information into collections of testable molecules. Therefore, unlocking small-molecule chemodiversity encoded in microorganisms, including the Fungi, will be crucial for next-generation drug development. Genome sequencing projects covering the whole spectrum of environmental fungi are providing fertile ground for directed secondary metabolite discovery. A variety of new approaches have revitalized the field of fungal natural product discovery, particularly in the fields of elucidation of biosynthetic mechanisms, identification of biosynthetic gene clusters and their connections to new and known fungal secondary metabolites, induction of homologous expression of repressed pathways in the native organisms, and heterologous expression of pathways in genetic model hosts. A valuable approach would be to focus community resources on generating high-quality sequenced genomes of all fungi that produce biologically important and structurally distinctive secondary metabolites and to connect these secondary metabolite clusters to their corresponding annotated gene cluster. Such a collective community project would streamline the recognition of orthologous pathways in new genomes and enable mapping of widely and narrowly distributed families of fungal metabolites. Concurrently, the discovery of novel secondary metabolite pathways would be accelerated. It would be logical to assume that at some point these new platforms for fungal natural product discovery would be introduced into industrial pharmaceutical and agrochemical discovery. Efforts to build industrial-scale genome-mining platforms for Actinomycetes are already under way (269). However, to the best of our knowledge, parallel efforts on the Fungi at an industrial scale have yet to be organized.

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