Soil Biology 36

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Genomics of Soil- and Plant-Associated Fungi

Soil Biology

Volume 36

Series Editor
Ajit Varma, Amity Institute of Microbial Technology,
Amity University Uttar Pradesh, Noida, UP, India

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ISSN 1613-3382
ISBN 978-3-642-39338-9 ISBN 978-3-642-39339-6 (eBook)
DOI 10.1007/978-3-642-39339-6
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013947369

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Printed on acid-free paper

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Foreword

Fungi, the "fifth kingdom" of living beings, comprise a wide variety of eukaryotic heterotrophs including mushrooms, rusts, truffles, morels, molds, yeasts, and many more families, genera, and taxa that are less well known. Their total number has been estimated to be in the range of 1.5 million species. Fungi have traditionally been considered to be microorganisms, because of the tiny hyphal filaments that are formed in laboratory cultivations. However, these hyphae can form extensive networks in their natural habitats, whose biomass is not smaller than that of large animals or plants.

Fungi have also developed numerous lifestyles: saprotrophs break down dead organic material and thus importantly contribute to nutrient cycling within the ecosystem. Yet many other fungi are biotrophs and form symbiotic associations with plants (mycorrhizae), algae (lichens), arthropods, and even prokaryotes. In fact, more than 80 % of the vascular land plants are obligatorily dependent on mycorrhizal symbioses with fungi. More recently, facultative and obligate endophytes of various plant tissues have also been discovered, and there is evidence that they can protect the plants against biotic and abiotic stress.

In order to be successful in their habitats, fungi have developed various successful strategies such as the ability to export hydrolytic enzymes to break down biopolymers or to produce chemicals that can antagonize competing organisms. Some of these properties have been exploited by humans for the production of goods for a long time: production of ethanol by the yeast *Saccharomyces cerevisiae* is the oldest example of the use of fungi for mankind and is experiencing a revival in this decade because of the interest in production of biofuels from renewable resources. Other 60-and-more-year-old examples are penicillin production by *Penicillium chrysogenum* and citric acid production by *Aspergillus niger*. These processes led to a more systematic exploitation of fungi and consequently the development of a number of biotechnological processes for the production of enzymes (such as cellulases, amylases, lipases, and glucose oxidase); platform

chemicals such as citric acid, itaconic acid, and gluconic acid; and numerous secondary metabolites that proved successful in medicine (e.g., penicillin, cephalosporin, cyclosporine, lovastatin). Due to the high secretory capacity that was detected in the enzyme producers, fungi have also been used as workhorses for the production of mammalian and plant proteins, which are only of limited availability from their native producer (e.g., chymosin). Finally, because fungi share a significant part of their gene repertoire with higher organisms, but can easily be studied and manipulated in the lab, some of them (e.g., *S. cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Neurospora crassa*) have extensively been used as model systems for basic biological processes such as the cell cycle, reproduction, or the circadian rhythm.

On the other side, many fungi also have negative impacts on mankind: the majority of contemporary plant diseases are caused by fungi and can achieve epidemic dimensions like the Dutch elm disease caused by *Ophiostoma ulmi* or chestnut blight by *Cryphonectria parasitica*. They also lead to large losses of nutritionally required crop plants by either affecting plant biomass formation (like the rice pathogen *Magnaporthe grisea*) or simply due to the secretion of mycotoxins (such as aflatoxin formation by *Aspergillus flavus*) and thus spoiling the harvest. Many other fungi can act as parasites of animals including humans, such as *Cryptococcus neoformans* that causes fungal meningitis. More recently, several fungi have proven to be opportunistic facultative pathogens, which do not target animals or humans regularly but can establish themselves in the body of immunocompromised individuals. In addition, many fungi have developed mechanisms to settle in the most extreme habitats and can occupy, e.g., building walls, thereby giving rise to "indoor contamination," or simply cause biodeterioration of materials, particularly manufactured wood.

In view of all these diverse activities, it is not surprising that fungi have also been intensively investigated towards detecting the mechanisms that form the basis of the various positive or negative effects. The final aim is to find tools that can be used either to increase their beneficial action or to combat their negative impact. This was, however, significantly impeded in those fungi where appropriate genetic systems to map mutations and cross mutants were unavailable. Today, the development of techniques to sequence, assemble, and annotate whole genomes and to functionally analyze their contents by systems biological approaches (transcriptomics, proteomics, metabolomics, and other "omic" technologies) has eliminated this bottleneck. The first major fungal genomics milestone was the publication of the whole genome sequence of the yeast *S. cerevisiae* 17 years ago. Today, more than 100 fungi had their genomes sequenced, and although this number is dominated by fungi of medical importance, genomic insights are already available for many other fungi. Today, we have arrived in several cases at first systematic insights into how fungi use their genetic repertoire for their specific behavior.

This book reviews the current state in our genomic understanding of those fungi which are primarily soil inhabitants and also those which interact with plants, whereby both positive and negative interactions will be covered. The comparison of their genomes reveals the various strategies, by which fungi use a basically similar repertoire of genes for differing purposes, and offers fascinating possibilities for future research.

Vienna, Austria Christian P. Kubicek

Preface

Fungi have a major role in natural ecosystems and in agriculture. This is particularly relevant for fungal species whose main niche is either soil, rhizosphere, plant roots, or above-ground plant tissues. The sequences of fungal genomes provide a new window to observe and understand how fungi recycle organic material in the soil, engage in positive and negative interactions with plant roots, and attack plants as pathogens. An unprecedented amount of sequence information has accumulated over the past decade, and researchers are now looking for ways to extract biological information. We assembled this volume in the hope that comparison across species will help bring focus to what is similar or different in the genomes of soil saprophytes, symbionts, and plant pathogens. There are now so many sequences that any attempt to catalog what is known for all fungi would require not one but tens of volumes and would quickly become out of date. Rather, we chose examples of species where particular principles can be illustrated. The reasons are diverse: from importance in ecology, agriculture, or medicine to model species that may have been convenient for applying a certain technique. If the approaches from one species eventually lead to fruitful work in another, we will be able to look back on a successful contribution to the science of fungi in its new genomic framework.

We are grateful to the authors of the chapters of this volume for all their thoughts and efforts. In particular, each chapter develops a unique approach that often reflects not only the fungal species studied but also the viewpoint, research priorities, and expertise of the research community studying each species. The fungal genome sequencing projects are the source of this book, which two decades ago would have been difficult to even imagine. Many of the contributors have been central participants in the genome projects or guiding future ones, and we hope to share the satisfaction of seeing a growing contribution of the genomics of soil, rhizosphere, and plant-interacting fungi to ecology, biotechnology, and sustainable agriculture. We would like to express our sincere thanks to the series editor Prof. Dr. Ajit Varma for supporting this project from the start and for his help in

bringing this volume to completion. It has been a pleasure to edit the volume, primarily due to the stimulating cooperation of the contributors. We wish to thank Hanna Hensler-Fritton and Jutta Lindenborn, Springer Heidelberg, for their generous assistance and patience in finalizing the book.

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April 30, 2013

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Chapter 1
Genomic Contributions to the Study of Soil and Plant-Interacting Fungi

Benjamin A. Horwitz, Prasun K. Mukherjee, Mala Mukherjee, and Christian P. Kubicek

1.1 Introductory Remarks

The first filamentous fungal genome published was of *Neurospora crassa* (Galagan et al. 2003), one of the classical genetic model systems (Davis and Perkins 2002; Perkins 1992). It is obvious, though, that not everything can be learned from the genome of a single species. Fungi are very versatile and degrade almost any substrate. The niche occupied by a given fungal species can be anywhere from very wide to very narrow. It was logical, therefore, to ask whether the genomes of plant pathogens would differ from saprophytes, and how. The sequence of rice blast as "the" model plant pathogen (Dean et al. 2005) indeed began to provide insight into what makes a pathogen different, with expanded families encoding signal transduction machinery, secondary metabolism, and possible virulence-related proteins. Later, though, the choice of species to sequence followed a more complicated path, guided by medical or agricultural importance, or by the activity of the research groups working on a particular group of fungi. Although different questions have been pursued in different species, there are unifying themes. Some of these themes are set by basic biology, for example, protein kinase genes related to the cell cycle, or the fundamental structure of the fungal cell wall. Others are related to interaction with other organisms: small secreted proteins that act as

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_1,
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virulence effectors for pathogens, effectors required to establish symbiosis, and enzyme repertoires that are tuned to a particular host or substrate.

1.2 Genetic Models

Neurospora crassa, as already mentioned, and *Aspergillus nidulans* are the most advanced model systems and are used to ask basic questions. *Neurospora* (years before its genome had been sequenced) was used to identify the clock gene *Frequency* (McClung et al. 1989) and the "white collar" blue light photoreceptor (Ballario et al. 1996). *Aspergillus* was established as a model of choice for developmental studies (Adams et al. 1988). Signal transduction schemes advanced for both *Neurospora* (Borkovich et al. 2004) and *Aspergillus* (Adams et al. 1998) are a standard for work on other species (see the discussion and schemes in Chaps. 5 and 7). As elsewhere in this volume, it is important to keep in mind work done in species that are not represented here. As an example, work on the mitogen-activated protein kinase (MAPK) pathway in fungi began with the finding that a MAPKK of *Ustilago maydis* is needed for conjugation tube formation, filament formation, filamentous growth, tumor induction on the maize host, and teliospore germination (Banuett and Herskowitz 1994). Rice blast is a premier model for the study of fungal signal transduction (Xu and Hamer 1996; Zhao et al. 2007). Model systems, though, can be looked at in a different light. The tools available for *Neurospora* (Chap. 3), for example, add a dimension to comparative work, where a well-understood process can be compared in many accessions. A quantitative trait loci (QTL) analysis of the circadian clock (Kim et al. 2007) identified 13 previously known clock loci but also 30 new ones. *Magnaporthe* is studied for the biochemistry (and physics) of appressorium development, but recent years have seen a novel above- and belowground view, addressing the question of what makes a pathogen attack either leaves or roots (Chap. 4).

1.3 Soil Fungi

Fungi have central roles in recycling organic material in the soil. The natural niche of the cultivated mushroom *Agaricus bisporus* is forest soil rich in humic substances, which consist of nonwoody, partially degraded plant material. The *A. bisporus* genome encodes a large set of carbohydrate-degrading enzymes enabling the fungus to break down polysaccharides from plant cell walls (Morin et al. 2012). In this respect, *A. bisporus* is similar to the sequenced wood-degrading Basidiomycetes (Chap. 13), but has relatively few lignin or manganese peroxidase genes (Morin et al. 2012). Soil inhabitants interact with the roots of living plants, and these interactions can be either beneficial, damaging, or somewhere in between. *Fusarium oxysporum* is the agent of vascular wilt, with a wide host range, but

exhibiting host specificity at the subspecies level. The analysis of the genomes of these fungi (Ma et al. 2010) revealed the presence of lineage-specific chromosomes wherein most of the pathogenicity-related genes are concentrated. Interestingly, horizontal transfer of these chromosomes rendered the nonpathogenic strains pathogenic (Chap. 7). Two species of *Verticillium*, related wilt pathogens, have also been sequenced, and their genomes encode an expanded capacity to make pectin-degrading enzymes (Klosterman et al. 2011). There is good reason to expect the genomes to help us understand why some species are pathogens and others beneficial to plants. Ancient symbioses (Remy et al. 1994) evolved together with land plants. Mycorrhizal fungi interact with roots in two very different ways, both of which result in exchange of minerals and nutrients between the partners. Plants and symbionts have evolved signaling mechanisms to establish their interactions (Oldroyd 2013; Oldroyd et al. 2009). The extensive hyphal network of the fungal mycorrhizal partner effectively increases the surface area of the root, transferring mineral nutrients (among which phosphate is a prominent factor) from the soil, and the plant provides carbon sources for the fungus. Furthermore, it was recently concluded from carbon isotope modeling that in boreal forests, in deeper soil layers, a large proportion of soil carbon compounds are derived from roots and associated mycorrhizal fungi, which thus have a central role in the sequestrating of carbon (Clemmensen et al. 2013). Endomycorrhizal fungi (AMF) penetrate root cells forming a distinctive intracellular network, the arbuscle (Bonfante and Requena 2011). The fungal members of this intimate symbiotic pair belong to the Glomeromycota; no species in this lineage has been sequenced yet, but an immense effort is under way. Moreover, transcriptomic (Gomez et al. 2009; Tisserant et al. 2012) studies and molecular genetic investigations (Kloppholz et al. 2011) of the mechanism of this important symbiosis are running ahead along with the genome project. Another type, the ericoid mycorrhizal fungi, belongs to several Ascomycete classes. There is a draft sequence for *Oidiodendron maius*, a member of the Leotiomycetes which can colonize the roots of some ericoid plants intracellularly (Joint Genome Institute). Ectomycorrhizae (EM) form an intercellular network in the cortex of the root (Plett and Martin 2011). EM fungi often belong to the Basidiomycetes, and the first sequenced EM species was *Laccaria bicolor* (Martin et al. 2008). *Paxillus involutus* is another well-studied EM Basidiomycete species, which has been sequenced to 36.2× coverage (Joint Genome Institute). Studies on *Paxillus involutus* are unraveling the molecular basis for how ectomycorrhizae act as both decomposers and mutualists. In particular, novel spectroscopic strategies combined with transcriptomics have now provided insight into the mechanism for how *P. involutus* obtains nitrogen by decomposing complex substrates in the soil (Chap. 8). There are also Ascomycete EM fungi, belonging to the classes Dothideomycetes (Chap. 9) and Pezizomycetes. The genome sequence of the truffle *Tuber melanosporum* represents the latter class; comparison with *Laccaria bicolor* shows that the set of genes enabling symbiosis has evolved differently (Martin et al. 2010; Veneault-Fourrey and Martin 2011). In contrast to *L. bicolor*, *T. melanosporum* does not have strikingly expanded gene families of small secreted proteins and might have some potential for breakdown of host cell

walls. *Trichoderma* spp. (Chap. 6) are mycotrophs that parasitize and destroy other fungi, commonly aboveground Basidiomycetes. They can also interact with plant roots and colonize the outer cortex layers in a way similar to EM, although it is debated whether root-interacting *Trichoderma* spp. can be considered as true symbionts. Similar to EM, *Trichoderma* spp. do not reach the vascular system, do not break down plant tissue, and do not cause disease. Nevertheless, the *Trichoderma* genomes encode enzymes for plant cell wall degradation which are needed to establish themselves in pre-decayed wood (Chap. 6). *Piriformospora indica* is a Basidiomycete root symbiont which interacts first as a biotroph, followed by a second phase characterized by local plant cell death which does not result in disease. The genome and transcriptome have some characteristics of biotrophs, for example, expression of genes encoding small secreted proteins, early in the interaction with roots (Zuccaro et al. 2011). Other species certainly can invade roots and cause disease, for example, the destructive soilborne pathogen(s) *Fusarium oxysporum* (Chap. 7). Comparison of these genomes should guide understanding of where symbiosis ends and disease starts. Finally, one must remember that myriad species have not been sequenced or even studied. Chapter 14 introduces the Archaeorhizomycetes, a novel group of soil fungi which is not yet represented among the sequenced genomes.

1.4 Pathogens and the Soil

Some soilborne fungi are serious pathogens of roots or of the entire plant, and several species have been sequenced. An isolate from the widespread Basidiomycete pathogen *Rhizoctonia solani* (isolated from a lettuce plant) was sequenced. The genome was characterized in detail (Wibberg et al. 2012), and impending functional analysis may shed light on the difference between pathogens and symbionts. Two widespread, destructive necrotrophic plant pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* persist in the soil, with the help of resistant sclerotia that develop from mycelia. This contrasts with some other members of the Leotiomycete class which are biotrophs on leaves. The sequences of *Sclerotinia sclerotiorum* and *Botrytis cinerea* provided an opportunity for comparative genomics (Amselem et al. 2011). The two species are close, and indeed, there was a high level of sequence identity and synteny. There are also some revealing differences and biological insight. *S. sclerotiorum* does not make conidia and relies on sclerotia alone for survival and eventual dispersal, while *B. cinerea* disperses by numerous small conidia, in addition to being able to survive as sclerotia in the soil. The genomes, though, did not provide an explanation for why *S. sclerotiorum* does not conidiate, since the relevant genes are present. Even though there were unique secondary metabolite clusters, there were no obvious unique virulence determinants, and regulation appears more important than the presence of sets of genes (Amselem et al. 2011).

The three pathogens just mentioned are bona fide soil residents, but the soil is a reservoir for some foliar plant pathogens whose spores are distributed above ground, for example, Southern corn leaf blight (Chap. 9). Thus, one can look above ground without going too far from the main emphasis of this volume, a case in point being rice blast and its relatives (Chap. 4). One of the plant pathogens whose molecular biology and genomics is most advanced is *Ustilago maydis* (Kamper et al. 2006). *U. maydis* was compared with the closely related *Sporisorium reilianum*. Among the salient findings from this study was that the effector repertoire has diverged much more than the average over the genome (Schirawski et al. 2010). If looking above ground, pathogens of humans are of vital importance. Three Zygomycete fungi, whose natural niche is dung and soil, have been sequenced: *Phycomyces*, *Mucor*, and *Rhizopus*. *Mucor* is one of many causes of opportunistic filamentous fungal mycosis (Perusquia-Ortiz et al. 2012). *Rhizopus* is an emerging pathogen (Chap. 11). The dermatophyte fungi are not normally life-threatening but cause immense suffering. The dermatophytes are completely adapted to life on the skin, rather than being opportunistic pathogens. Soil saprophytic, geophilic species are closely related to those causing disease on human or animal hosts, and when five dermatophyte species including the geophyte *Microsporum gypseum* were sequenced and compared, the genomes were highly collinear. There were also genes unique to each species, but signaling and regulation of gene expression may play the major role in determining host and niche (Martinez et al. 2012).

1.5 Contribution of Genomics: Progress and Outlook

Genomic sequences provide an unprecedented view into the diversity of fungi that reside in the soil or spend part of their life cycle there and, of course, into their relatives that have no association with soil. A recurring motif in the published genome papers and in the chapters of this volume is that, sometimes, analysis of the genomes directly answers a biological question. Biochemical analyses, though, are often needed to go from genome to enzyme activity and function. When a group of fungi was long known for a particular function, there is abundant biochemical literature, but less so for species where new biochemical potential was found to be encoded in the genomes. An example is lignin degradation by rot fungi of the Agaricomycetes (Chap. 13), while the potential exists in Ascomycetes (Chap. 12), where new biochemical questions can now be asked. *Aspergillus* spp. are the classical models in which to study secondary metabolites, while the newly discovered biosynthetic clusters in other species, some of them discussed here, are leading chemists into new territory (Brakhage 2013; Lim et al. 2012). In other analyses, huge efforts led to an understanding of genome structure, phylogeny, and evolution, but cannot yet give a full answer as to why one species is a pathogen while a related one is a saprophyte, or mutualist. There are many hints in the chapters that follow to suggest that regulatory networks may provide the answers. From the technological

point of view, the genomes are the first step, prerequisite to any attempt at understanding these regulatory networks.

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Chapter 2
Fungal Genomics for Energy and Environment

Igor V. Grigoriev

2.1 Introduction

The finite resource of fossil fuels and the adverse ecological impacts of their exploration and use pose significant challenges for mankind. Development of alternative energy sources goes hand in hand with restoring ecological balance. This requires knowledge of how natural biological systems work. One such system includes fungi that interact with plants as symbionts, pathogens, or decomposers. These interactions, first, determine growth of biomass and, second, provide clues toward efficient conversion of plant-produced lignocellulose into energy, offering real alternatives to fossil fuels. Understanding these processes at the molecular level is therefore a critical challenge.

The tools of molecular biology and genomics can help us meet this challenge. Over the past decades, genomics, and genome sequencing in particular, have emerged as powerful tools for biological research. In the last few years, due to the introduction of the next-generation sequencing (NGS) technologies, these tools went through dramatic transformations. The first genome projects like sequencing the genome of *Saccharomyces cerevisiae* were colossal multi-institutional, multinational sequencing efforts (Goffeau et al. 1996), which reached their culmination with the Human Genome Project (Lander et al. 2001). Though deemed an extremely large quantity of data several years ago, the 3×10^9 base pairs of the human genome represent only a fraction of the data produced from just a single lane of an Illumina sequencer these days. Genomics technologies are thus poised to help us study not just individual organisms but entire ecological systems.

With technology breakthroughs, the scope of the projects has evolved accordingly. Genomics projects have reached an unprecedented scale like the 1000 human genomes (2010) or ENCODE (2004) and enable scientists to ask new types of

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_2,
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questions. How can genomics help to obtain a sustainable growth of biomass? What is the role of microbial association with plants? Do genome features determine if a fungus is a friend (symbiont) or a foe (pathogen)? How do microbes efficiently convert biomass into energy? What biological mechanisms govern ecological balance? Here, in the context of fungal genomics for energy and the environment, we discuss tools, applications, and the most recent developments available to explore the biology of soil fungi at the molecular level.

2.2 The Tools of Genome Sequencing

The first sequencing experiments were very laborious. Not long ago, a scientist would have to make a significant effort to sequence a single gene. At the end of the twentieth century, Sanger sequencing (Sanger et al. 1977) became the dominant way to sequence genomes, including the human genome. At the beginning of the twenty-first century, suddenly several new NGS platforms were introduced (Metzker 2010). First, 454 (now Roche) offered a new technique called pyrosequencing as a way to read DNA fragments in a high-throughput fashion and for just a fraction of the cost of Sanger sequencing. Shortly after, Solexa (later acquired by Illumina) developed a new way to produce very large numbers of very short (initially 25 bp) reads at a much less cost. More recently, Pacific Biosciences presented a single molecule sequencing approach to produce long reads but with a relatively high error rate (up to 15 %). These are just a few among the larger collection of sequencing platforms that are available today. Each of them dramatically improved characteristics of the produced sequence reads—length, error rate, throughput, and GC bias—during just the last few years. Innovation continues as new players like Oxford Nanopore promise groundbreaking solutions in the near future (Pennisi 2012).

Many sequence analysis tasks have been solved in the era of Sanger sequencing. Genome assemblers like Arachne (Batzoglou et al. 2002) are capable of putting together Sanger reads into assemblies for both small bacteria-size and large plant-size genomes. However, some sequencing and analysis problems remain quite challenging. Sequencing shows some platform-dependent bias. Repetitive sequences make it difficult to place reads uniquely into an assembly. Polymorphism and polyploidy interfere with clean separation of haplotypes.

A few dozen fungal genomes were sequenced using the Sanger platform and have draft assemblies available in public databases like GenBank (http://www.ncbi.nlm.nih.gov/genbank). Using multiple iterations of targeted Sanger sequencing, many gaps in several draft genomes were closed in a process called genome finishing. This resulted in at least a dozen small yeast-size finished genomes (Dujon et al. 2004) starting with *S. cerevisiae* and only a couple of finished genomes of filamentous fungi (Berka et al. 2011; Goodwin et al. 2011). Many others including the model fungus *Neurospora crassa* went through multiple rounds of

genome improvements but continue to keep "secret messages" in some as-yet unresolved parts of their genomes (Galagan et al. 2003).

Genes encoded in the sequenced and assembled genomes can be predicted and functionally annotated using different computational approaches (Grigoriev et al. 2006; Haas et al. 2011). The complex intron-exon structure of a eukaryotic genome makes annotation a challenge in comparison to the simpler problem of calling ORFs in compact, gene-dense prokaryotic genomes (in which genes typically lack introns). Eukaryotic gene prediction usually combines several methods including transcriptome-based (deriving genes from ESTs mapped to a genome assembly), homology-based (based on proteins from other genomes mapped to a translation of the assembly), and ab initio gene predictors. For the latter, which sometimes is the last resort (since preference is usually given to genes predicted from experimental data or similarity to known proteins), gene structure features are derived from a collection of known genes and then are searched for in the entire genome to predict new genes using these features. Gene structures or models predicted using these methods also require annotation methods to predict possible gene functions. For a given protein encoded in a genome, function can be inferred from known proteins or protein domains if their protein sequences are sufficiently similar as determined by various alignment programs like BLAST or HMMER (Altschul et al. 1990; Bateman et al. 1999). The problem is that despite the quickly growing number of sequenced genes, the number of biochemically characterized proteins grows very slowly. In addition, computational methods for gene prediction and annotation as well as reference databases themselves are error prone. Therefore, both structural and functional annotations are often followed by manual inspection in which trained analysts look at genomes, predicted genes and available lines of evidence in genome browsers. The largest scale efforts in manual curation have been achieved for the human genome, although several model fungi including *S. cerevisiae*, *S. pombe*, *and N. crassa* also have curators devoted to iterative improvement of these datasets (Howe et al. 2008).

The new sequencing technologies have brought new types of data, for example, very short reads in large numbers from Illumina or longer and error-prone reads from 454 and Pacific Biosciences sequencers. Variation in read sizes and numbers demands completely different analytical approaches. Various implementations of de Bruijn graph have been used in new assemblers such as Newbler, Velvet, and AllPathsLG that were tuned for different platforms (Earl et al. 2011). Hybrid sequencing and assembly has become the norm, with assemblies often being constructed from Illumina, 454, and Sanger reads all pooled together, often with different assembly algorithms used for the different kinds of reads. However, even though NGS was much cheaper than Sanger sequencing, the resulting genomes are generally of lower quality. To compensate for this, the questions posed and the applications of these platforms were adjusted accordingly. For example, NGS has given rise to massive re-sequencing and transcriptomics applications. Annotation methods have changed as well using RNA-seq data as a primary source of data.

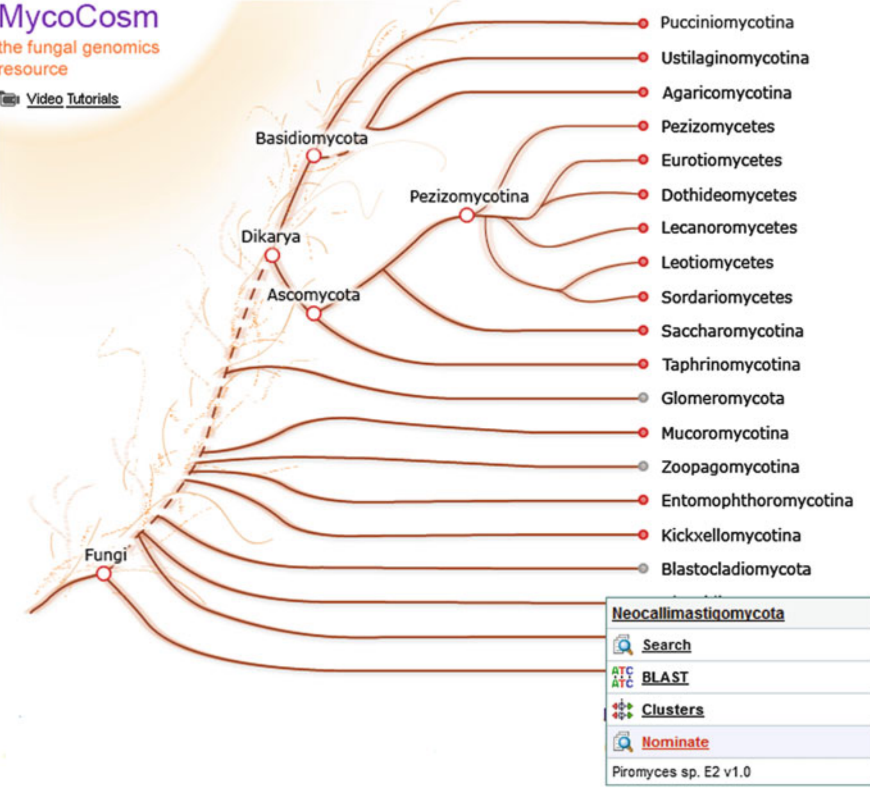

Fig. 2.1 JGI fungal genome portal MycoCosm (http://jgi.doe.gov/fungi) with 200+ fungal genomes and tools for their comparative analysis and nomination of new fungal species for sequencing

Data integration and visualization problems formulated during the human genome era from the need to put genome sequences, gene predictions, ESTs, and protein homologs on one screen became even more complex when confronting massive amounts of sequence data and large numbers of sequenced genomes. While GenBank offers a large collection of sequenced genomes and many bioinformatics tools are open access, the lack of their integration makes them difficult and time-consuming to use. One solution has been developed specifically for fungal genomes by the US Department of Energy (DOE) Joint Genome Institute (JGI): the web-based fungal genome portal MycoCosm, which offers 200+ fungal genomes and tools for their comparative analysis and manual curation (Grigoriev et al. 2012; Fig. 2.1).

2.3 Plant–Microbe Interactions and Evolution of Fungal Lifestyles

The Human Genome Project started a revolution in health care. Similarly, plant genomics became a game changer in plant breeding. The poplar genome (Tuskan et al. 2006), for example, led to research investigating the role of various transcription factors on plant growth. However, the genome only reflects the organism's potential to develop, while the actual growth and development depends on interaction with the environment including microbial interactions. Fungal symbionts and pathogens are important players in these interactions. The majority of plant species are dependent on mycorrhizal associations. Pathogens can destroy a significant share of agricultural and bioenergy crops like the Southern corn leaf blight, which in the 1970s destroyed the entire corn crop in several US states. Decomposers recycle dead materials to provide nutrients for the new generations of primary producers and microbes associated with them. These lifestyles—symbiosis, pathogenicity, and saprotrophism—are encoded in genomes. Thus, genome analysis and comparison of different genomics features are essential for understanding fungal lifestyles, their evolution, and interactions to possibly lead to better management practices.

Genomes of several organisms involved in interactions with each other in natural ecosystems have been sequenced. One such system includes a poplar tree and associated ectomycorrhizal symbiont *Laccaria bicolor* and pathogenic rust *Melampsora laricis-populina*. Interestingly, genomes of these symbiotic and pathogenic fungi share several things in common: large genomes inflated with repeats and expanded gene families, the most interesting of which is a large number of small secreted proteins (Martin et al. 2008; Duplessis et al. 2011). Despite being very abundant, small secreted proteins are frequently lineage specific. They may share some functional domains between symbionts and pathogens but hardly show any sequence similarity even between closely related poplar rust and wheat rust. Interestingly, in all these fungi, genes encoding the small secreted proteins are among the most expressed in planta, during infection of the plant host.

Another important part of both plant symbiont and pathogen gene sets is CAZymes, the carbohydrate-active enzymes (Cantarel et al. 2009) involved in lignocellulose degradation, fungal cell wall reconstruction, and other important processes. In contrast to pathogens, whose expanded CAZy families aim to modify and destroy the host plant, the genome of symbiotic *L. bicolor* contains a relatively limited arsenal of CAZymes and a lack of those involved in plant cell wall degradation, which results in a protection mechanism to minimize the impact on the plant host. The symbionts thus evade the plant's defense responses (Martin et al. 2008). Interestingly, this reduction is similar to the reduced CAZy profiles of one group of saprobic, wood-decaying fungi called brown rot fungi. In contrast to the white rot fungi (the second and dominant type of wood decay), brown rot fungi have evolved to employ a less "expensive" mechanism for lignocellulose degradation (Eastwood 2011; Martinez et al. 2004, 2009). Instead of enzymatic attack typical for white rot fungi, brown rot relatives are thought to use Fenton chemistry

to generate highly reactive hydroxyl radicals to break cellulose chains. They also do not degrade lignin and thus lack the corresponding genes in their genomes.

For some wood decayers, it is not always obvious if white or brown rot is their natural mode of decay (R. Blanchette, pers. comm.) and their genomes may provide some hints. On the other hand, the genome of *Agaricus bisporus* apparently encodes enzymes involved in lignin degradation, though the fungus has not been observed to do so in nature (Morin et al. 2012). Fungi efficiently combine different lifestyles. *L. bicolor* as a saprobe extracts nutrients from decaying organic matter to provide them to the host plant, with which it also forms mycorrhizal association. The white rot fungus *Heterobasidion irregulare*, also a pathogen of conifers and other trees, encodes both of these lifestyles into its genome and balances between these lifestyles even when growing on the same host (Olson et al. 2012). Thus, interactions between plants and fungi are complex, and lifestyles of members of these interactors—symbionts, parasites, and saprobes—are hard to define with clear boundaries. A better understanding of the genomics basis of different lifestyles will require more complex analyses and large-scale comparative genomics studies.

2.4 Large-Scale Comparative Genomics

Discoveries based on the analysis of individual genomes become stronger in the context of comparative analysis. Instead of sequencing genomes one after another, analysis of groups of phylogenetically divergent fungi that share common traits or lifestyles may enable mapping of these traits to a specific set of genes and genomics features. JGI is one of the institutes partnering with numerous scientific groups around the world to explore the diversity of fungi, which are important for solving energy and environmental problems. Starting with the first sequenced basidiomycete, the white rot fungus *Phanerochaete chrysosporium* in 2004, by 2012 JGI has contributed to over a half of all fungal genome projects worldwide. After delivering several "first of its kind" fungal genomes—wood decayers, ectomycorrhizae, and thermophiles—JGI launched a project called the Genomic Encyclopedia of Fungi (Grigoriev et al. 2011) devoted to several areas of plant health and biotechnological applications for energy and the environment, the DOE mission areas. By 2012, the first two chapters of the encyclopedia, the large-scale comparative genomics studies, were published (Floudas et al. 2012; Ohm et al. 2012), while sequencing for several others was nearly complete.

Understanding the mechanisms of lignocellulose degradation by wood-decay fungi is important to finding new ways for processing biomass into biofuels. The first sequenced white and brown rot fungi, as mentioned earlier, revealed completely different mechanisms of wood decay encoded in their genomes and justified more extensive sequencing of this group of fungi. About 30 species of wood-decay fungi were selected for sequencing at JGI, and recently 12 of them were analyzed and reported in the context of 31 other sequenced fungal genomes (Floudas et al. 2012). This work has catalogued the largest collection of genes

encoding CAZymes while focusing on the analysis of the class II peroxidases involved in lignin decay. The ancestor of both white and brown rot fungi (as well as mycorrhizal *L. bicolor*) was capable of processing lignin, which was produced by plants and converted into coal in prehistoric times. Molecular clock analysis suggested that the white rot ancestor evolved approximately at the end of Carboniferous, i.e., potentially in time to contribute to the significant decline of coal accumulation observed at that time. In other words, what could have contributed to the decline in fossil fuel accumulation ~300 Mya can help us today to make progress in developing biofuels.

In order to convert biomass into biofuels, the former needs to be produced in a sustainable fashion. Pathogenic fungi are notorious for destroying a significant fraction of agricultural crops and can destroy bioenergy crops to the same or greater extent. In order to protect plants, we should better understand the molecular basis of different strategies of pathogenicity. The Dothideomycetes are an example of a diverse class of fungi that contains a large number of plant pathogens. Several independent research groups at various genome centers have been sequencing fungal genomes from this class and converged in 2008 at JGI to consolidate the genomics data for comparative analysis and propose a much larger set of sequencing targets. As a result of this effort, 14 newly sequenced Dothideomycete genomes were compared with each other and fungi sequenced earlier to explore different modes of pathogenicity and patterns of their evolution (Ohm et al. 2012). This revealed common features of genome organization across the entire class: an inversion-based mechanism for mesosynteny or gene reshuffling within the boundaries of chromosomes; a variable number of dispensable chromosomes with unclear role in pathogenesis; and blocks of genes conserved in most of these species and expressed during plant infection in some of them. Gene family expansions and contractions were traced along the evolution of major groups of Dothideomycetes, Capnodiales, and Pleosporales and revealed larger sets of genes involved in secondary metabolism and plant cell wall degradation in necrotrophs vs. biotrophs with stealth pathogenesis like *Mycosphaerella graminicola* (Goodwin et al. 2011). This global genome comparison was followed by several functional studies focused on specific gene families (Condon et al. 2013), plant-pathogen systems (Manning et al. 2013), and functional platforms (Cho et al. 2012). One of them suggested that differences in gene regulation may be the key in determining host specificity even in very closely related species such as *Dothistroma* and *Cladosporium fulvum* (de Wit et al. 2012) and that functional genomics would be the next critical step in understanding fungal biology.

Besides the Agaricomycetes and Dothideomycetes, large-scale comparative analysis of other groups of fungi has been progressing quickly: these include 30+ mycorrhizal fungi (Chap. 8), 20+ yeasts of biotechnology and taxonomic importance, and 10+ species of *Aspergillus* and *Penicillium* for various biotech applications (Chap. 5). Finally, the desire to ask bigger questions through larger scale sequencing transformed one of the chapters, fungal diversity, into a project of unprecedented scale: the 1000 fungal genome project.

2.5 The 1000 Fungal Genome Project

Advances in genome sequencing have allowed scientists to launch very large-scale genomics projects like 1000 human genomes (2010), 1001 *Arabidopsis* genomes (Weigel and Mott 2009), and GEBA (Wu et al. 2009). *The 1000 fungal genome projec*t is one of the latest JGI large-scale genomics initiatives aimed at highly divergent fungal species to obtain a comprehensive list of reference genomes, to better assess fungal diversity, to explore evolutionary processes driving this diversity, and to provide a comprehensive vocabulary for studying complex metagenomes.

The Kingdom Fungi is estimated to contain over a million species. These organisms developed a tremendous natural arsenal of enzymes, chemicals, deconstruction, and synthesis mechanisms over millions of years of evolutionary history, which are poorly understood. Despite the growing number of fungal genome sequencing projects, the phylogenetic diversity of fungi covered by these projects is still very limited. Ascomycetes of medical importance remain dominant among the sequenced fungal genomes. In contrast, lower fungi are hardly represented among the currently available reference genomes.

The goal of the 1000 fungal genome project is to sequence genomes for on average two species for each of the about 500 known fungal families within 5 years. The project started in close collaborations with several culture collections and research groups providing DNA and RNA samples. JGI accepts nominations for new species for sequencing and DNA/RNA samples from the scientific community worldwide at http://jgi.doe.gov/fungi (Fig. 2.1). These will serve as references in ecological genomics.

2.6 Ecological Genomics

Having a large collection of reference genomes may set a stage for eukaryotic metagenomics. Metagenomes of bacterial and archaeal communities have been successfully analyzed previously (Tringe and Rubin 2005; Kalyuzhnaya et al. 2008). Even when metagenomes are poorly assembled but dominated by prokaryotes, the assembled pieces provide sufficient information to predict genes. Unlike gene-dense bacterial genomes, eukaryotic genomes, with their complex gene structure and genome organization, present a significant bottleneck for metagenomics. Assembled DNA pieces lack sufficient information to train ab initio gene predictors. Homology-based methods may work but require a representative collection of reference genomes. While this collection is being built over time, approaches to assess complexity of fungal communities, for example, in soil, are being explored (Buée et al. 2009).

A standard method to identify fungal species is by their Internal Transcribed Sequences (ITS). Targeted ITS sequencing can be applied to fungal communities

composed of multiple species to assess their composition. Here transition to the NGS imposes some challenges. In the days of Sanger sequencing, long sequence reads would cover the entire region which is ~1 kb long. The first NGS products were too short to cover the entire ITS, although several studies used 454 pyrosequencing to obtain ITS fragments, ITS1 or ITS2 regions (Buée et al. 2009). The latest generation of Illumina machines, the benchtop MiSeq, offers sequencing in the format of 2 × 250 bp reads, which with sufficiently short inserts may overlap and produce contigs long enough to cover one of these regions. The cycle time of these machines (1 day instead of 18 for HiSeq) allows sequencing a multitude of samples.

Another strategy to overcome the complexity of fungal gene structure is metatranscriptomics, which gives a functional portrait of the community as a biological system and captures its dynamics. The challenge is the poly (A) enrichment in complex communities like soil where fungi make up just a few percent of the entire microbial transcriptome. Furthermore, total eukaryotic RNA consists of only a few percent of mRNA.

2.7 Functional Genomics

The increased throughput in genome sequencing has created a situation where the number of sequenced genes and genomes grows dramatically each year but does not necessarily help us to better understand their functions. A thorough biochemical characterization is required to determine gene functions, but its throughput is not on par with sequencing.

Analysis of gene and protein expression under different conditions may suggest roles of these genes in an organism's growth, while genes' co-regulation can be inferred from patterns of their co-expression. Large-scale transcriptomics has been broadly successful for a number of fungi and quickly progressed from in-depth characterization of genes in single species to multispecies comparative functional genomics, as with fission yeasts (Wilhelm et al. 2008; Rhind et al. 2011). Proteomics of different flavors offer approaches complementary to gene expression analysis and allows the characterization of proteins, protein complexes, and post-translational modifications. Among fungi, this was applied to the greatest extent to *S. cerevisiae* (e.g., Ho et al. 2002; Ptacek et al. 2005; Krogan et al. 2006). The combination of transcriptome and proteome analyses is becoming more and more often a part of genomics studies of many fungi (e.g., Berka et al. 2011; Martinez et al. 2009).

The roles of genes can be also determined by turning genes on and off using various techniques at reasonably high throughput. Transcriptomics and proteomics studies of gene deletion mutants, especially transcription factors, can point to their roles in an organism's regulatory cascades. Several studies along these lines have been done for fungi. For example, novel virulence factors have been identified in the plant pathogen *Alternaria brassicicola* (Cho et al. 2009, 2012), factors

influencing cellulose production have been studied in the industrial workhorse *Trichoderma reesei* (Schuster et al. 2012), the role of transcription factors has been explored for mushroom development in the basidiomycete *Schizophyllum commune* (Ohm et al. 2010, 2011), and a much broader approach is taken in the model ascomycete *Neurospora crassa* (Colot et al. 2006). For the models *N. crassa* and *S. cerevisiae*, extensive collections of deletion mutants along with microarrays and other functional genomics resources have been developed (Winzeler et al. 1999; Giaever et al. 2002; Dunlap et al. 2007), which created a solid basis for future experiments.

A different context for gene function studies comes from the analysis of interactions of fungi with other organisms. Analysis of the transcriptome at different stages of plant infection (O'Connell et al. 2012) or of interactions with a fungal prey (Atanasova et al. 2013) has been revealing for the dynamics of such interactions. However, looking at both partners at once can give more complete and, therefore, more accurate picture. Indeed, transcriptomes of the fungus *L. bicolor* and poplar tree upon their interaction provided clues for a metabolic model of nutrient exchange between them (Larsen et al. 2011). For pathogens, Skibbe et al. (2010) have shown that infection of maize by corn smut (*Ustilago maydis*) depends on organ-specific gene expression by both host and pathogen. Proteomics of such interactions was also studied in several different systems (reviewed by El Hadrami et al. 2012). Much larger-scale transcriptomics studies of several host-pathogen and mycorrhizal systems are also currently in progress at JGI. Finally, along the lines of the human ENCODE, which recently generated a very large amount of functional data (Skipper et al. 2012), *N. crassa* is a target of fungal ENCODE at JGI to further understand this model organism and project this knowledge to other fungi (Chap. 14).

2.8 Conclusion

Fungal diversity is enormous and so far poorly explored. Soil is the most abundant ecosystem on Earth, enriched in microbial life including a large number of fungal species. Very few microbial species inhabiting soil have been characterized. Genomics and transcriptomics offer new ways to identify these poorly characterized species and understand their function and interactions with environment, hosts, and other fungi. Metagenomics approaches can help to better understand the complexity of microbial communities in soil, how they are formed, and how they change in response to various environmental factors. Communities of pathogens and symbionts are components of the rhizosphere and determine the success of plant growth. Genomics analysis of these interactions will help us to better understand natural biological systems and can lead to applications for environmental protection and bioenergy production.

Acknowledgments The work conducted by the US Department of Energy Joint Genome Institute is supported by the Office of Science of the US Department of Energy under Contract No. DE-AC02-05CH11231.

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Chapter 3
Advancement of Functional Genomics of a Model Species of *Neurospora* and Its Use for Ecological Genomics of Soil Fungi

Kwangwon Lee and John Dighton

3.1 Resources for the Functional Genomics

High-quality genome sequence and annotation of the genome is a key element for successful functional genomics study. Since the release of the *N. crassa* genome sequence in 2003 (Galagan et al. 2003), there were several updates in sequence assembly and annotations. As of June 2010, the finished genome has 9,733 protein-coding genes, 176 noncoding RNA genes annotated in the nuclear genome, and 28 protein-coding genes in the mitochondrial genome (http://www.broadinstitute.org/annotation/genome/neuospora). As of November 2011, a whole-genome knockout project generated 11,721 knockout strains (http://www.dartmouth.edu/~neurosporagenome/knockouts_completed.html) (Dunlap et al. 2007). All the knockout strains are available at the Fungal Genetics Stock Center (http://www.fgsc.net/ncrassa.html). As part of the Neurospora Genome Project, a group of undergraduate students have cataloged ten different developmental phenotypes of the knockout strains (http://www.broadinstitute.org/annotation/genome/neurospora/Phenotypes.html) (Turner 2011).

These genetic resources in the *Neurospora* community are useful for generating a hypothesis for testing potential biological functions of an uncharacterized gene in a less characterized fungal species. The knockout library also allows for screening genes that are involved in a particular biological function of interest (Fu et al. 2011; Hammond et al. 2011; Nargang et al. 2012; Watters et al. 2011). Instead of screening the whole knockout library, one might choose to work on the smaller set of knockouts of transcription factors (Sun et al. 2011b) and kinases

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*,
Soil Biology 36, DOI 10.1007/978-3-642-39339-6_3,
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(Park et al. 2011) to identify key regulators for phenotypes of interest. Once the list of key regulators is identified in *N. crassa*, one might study the homologues of those regulatory genes in other fungal species that are more relevant for soil ecology.

The mutant with a complete knockout of a gene does not always reveal all biological functions of the gene. Diverse allelic phenotypes of a gene provide more rich information of the roles of the gene. For example, one of the key circadian clock genes, *frequency*, has different clock phenotypes depending on where in the protein-coding region a mutation occurred; frq^7 shows a 29 h long period, frq^1 16 h short period, and frq^{KO} no rhythm (Heintzen and Liu 2007). Cloning and characterizing the molecular nature of the mutation have become easier as a result of technical advances in sequencing. In recent years, there have been a series of improvements in identifying genome-wide single nucleotide polymorphism (SNP) markers utilizing microarray chips and next-generation sequencing (NGS) for the *N. crassa* genome (Baird et al. 2008; Dettman et al. 2010; Lewis et al. 2007; Rowe et al. 2011). Restriction-site-associated DNA (RAD) markers rely on SNP at the restriction enzyme recognition sites in the genome. Since developing RAD markers does not require high-quality backbone sequencing data, and as the cost for NGS sequencing is becoming reasonable, RAD markers have the potential to be used for mapping and cloning fungal genes in non-model soil fungi.

There are useful tools developed for characterizing the expression of a gene and the gene product in *Neurospora*. Although luciferase reporters have been successfully used in other organisms for studying real-time gene expression, this system has been less successful in fungal species. Dunlap's group developed an efficient luciferase reporter system by synthesizing the firefly luciferase based on optimal fungal codon usage (Gooch et al. 2008). Combining with a clever knock-in approach, one can also study protein expression at the endogenous genome location in real time (Larrondo et al. 2012). For biochemical studies, one has to express the protein of interest. There are a series of versatile vectors available for expressing proteins with commercial epitope tags (Honda and Selker 2009) with tight regulation of their expression (Hurley et al. 2012).

3.2 Sensing Light

3.2.1 Introduction

Microorganisms can detect a variety of signals from the environment, including light, temperature, gravity, and large or small molecule signals, often coming from the neighboring species in the environment (Corrochano 2007; Elias-Arnanz et al. 2011; Rockwell and Lagarias 2010; Rodriguez-Romero et al. 2010; Sharrock 2008). Responses to these signals may be to relocate to a favorable location, for orientation, to alter gene expression and development, or to deploy small molecules to cope with environmental conditions or survival in their host (Bahn et al. 2007;

De Sordi and Muhlschlegel 2009; Rockwell and Lagarias 2010; Roux et al. 2009). Light is probably the most central of all environmental signals. Thus, microorganisms have evolved sophisticated light-sensing mechanisms (Rodriguez-Romero et al. 2010; Sharrock 2008). For example, light affects many developmental transitions in fungi; it sets the phase of circadian rhythms and modulates the rate and direction of growth. The blue end of the visible spectrum including the near ultraviolet is most active, although longer wavelengths are also important in some species and responses, and filamentous fungal genomes encode a variety of known, or potential, photoreceptors (Bahn et al. 2007; Corrochano 2007; Purschwitz et al. 2008). Much of what we know about molecular photophysiology in microorganisms has been learned by studying a few selected model organisms, for example, in the fungal kingdom, *Neurospora*, *Aspergillus*, *Fusarium*, and *Phycomyces* (Avalos and Estrada 2010; Bayram et al. 2010; Chen et al. 2010; Corrochano and Garre 2010; Rockwell and Lagarias 2010; Roux et al. 2009). Also the interaction between light, microbial communities and litter decomposition in natural ecosystems appears to be synergistic, although the mechanisms of interaction are not completely understood (Gallo et al. 2009).

3.2.2 Neurospora

Neurospora is the best-studied microorganism in molecular mechanisms of light perception, signal transduction, and light-regulated development. In *Neurospora*, all known light-induced phenotypes are regulated by blue light, and all blue-light-induced phenotypes are absent in either *white collar-1* (*wc-1*) or *white collar-2* (*wc-2*) mutants (Ballario and Macino 1997). Attempts to find loci other than *wc-1* and *wc-2* have failed (Degli-Innocenti and Russo 1984; Linden et al. 1997), suggesting that WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2) are the major nonredundant key components for blue-light transduction in *Neurospora*. The *wc-1* and *wc-2* genes have been cloned (Ballario et al. 1996; Linden and Macino 1997), and their products have been shown to interact with each other (Ballario et al. 1998; Cheng et al. 2001; Denault et al. 2001). These findings and more recent molecular studies showed that WC-1 and WC-2 are transcription factors mediating light-induced gene expression (Corrochano 2007). Both WC-1 and WC-2 are regulated posttranslationally through phosphorylation (Lee et al. 2000; Schwerdtfeger and Linden 2000, 2001; Talora et al. 1999). While light-induced phosphorylation of WC-1 is transient (Heintzen et al. 2001), phosphorylation of WC-2 is stable under constant light. WC-1, working with WC-2 and the cofactor FAD, has been confirmed to be a blue-light photoreceptor (Froehlich et al. 2002; He et al. 2002).

3.2.3 Soil Fungi

The importance of light conditions for microbial development has been well documented. However, there is a lack of understanding of the roles of ambient light at the community level, more specifically in the context of interspecies and inter-kingdom interactions. Light could be an important factor determining community assemblages on surfaces exposed to light, for example, leaf surfaces. The enhanced frequency of melanin-containing fungi in high-light environments has led to investigations into the properties of melanin in fungal cell walls. For example, melanized cell walls are thought to protect cell contents from UV light (Gauslaa and Solhaug 2001) and may be a protectant against ionizing radiation (Durrell and Shields 1960; Gauslaa and Solhaug 2001; Zhdanova et al. 1994). Melanin has been shown to be an agent for entrapment of ionizing radiation for transfer of energy from radioactive sources for fungal growth (Dadachova et al. 2007; Dadachova and Casadevall 2008; Dighton et al. 2008; Tugay et al. 2006). Additionally, there is a tentative suggestion that light regimes can also influence soil fungal growth (Karpenko et al. 2006). Such responses of fungi to both light and ionizing radiation warrant further research, especially an understanding of the molecular basis of their function. As in the case of extensive light regulation studies in *Neurospora* (as discussed earlier), blue-light regulation is the most prominent light regulation in fungi. Thus, one might ask: “Do soil fungi have blue-light receptors, e.g., WC-1 orthologous genes, and could their presence influence the depth distribution of fungi in the soil profile?”

Understanding the response of the microbial community to a specific wavelength of light will provide a valuable insight into its adaptation to the ambient local environment. For example, plant leaves absorb photosynthetically active radiation (PAR, 400–700 nm) and transmit far-red/near-infrared radiation (700–1,000 nm) (Kume et al. 2011). Thus, the microbial community under a forest canopy is constantly exposed to higher flux densities of far-red light. It is tempting to speculate that the microbial community in the open field and that in the forest canopy are adapted to these unique light conditions. The differential response of fungal taxa to light of contrasting wavelength may be important for regulating community assembly and function of litter-dwelling fungi.

3.3 Lignocellulose Metabolism

3.3.1 Introduction

Biofuels derived from lignocellulosic biomass are a promising means of fulfilling the crucial need for liquid transportation fuel not derived from fossil fuels or food crops (DOE Office of Biological and Environmental Research). Lignocellulosic biomass, which is composed of cellulose, hemicellulose, and lignin, forms woody,

fibrous plant materials such as stems and leaves and is the most abundant renewable biomass on earth (Eriksson et al. 1991; Sanchez 2009). Unfortunately, utilizing lignocellulosic biomass as a biofuel feedstock is a challenging proposition owing to the difficulty of degrading lignocellulose in comparison to simple sugars, and current processes for producing cellulosic biofuel are not cost-competitive with fuels derived from either fossil fuels or food crops. While current industrial processes for degrading lignocellulose are too inefficient to be commercially viable, some living organisms are proficient at the task. In particular, a number of microorganisms have been identified that are capable of breaking down complex plant biomass and can potentially be used in industrial fermentation (Wilson 2008). For example, diverse fungal organisms in two phyla, Ascomycota and Basidiomycota, are capable of degrading lignocellulose (Eriksson et al. 1991; Lundell et al. 2010). Understanding the enzymes and metabolic pathways that provide this capability, particularly at a systems level, has been identified as a fundamental technical barrier that needs to be addressed to realize the goal of cost-competitive cellulosic biofuel. Additionally, some of the potentially useful fungal strains for biofuel production are slow growing, so there may be interest in transposing specific enzyme-coding genes from these fungal species into strains that grow more readily in a biofermentor environment.

3.3.2 Neurospora

Neurospora has been described as a "burning-adapted" fungus since the majority of the natural isolates were collected from the fresh-burn sites (Jacobson et al. 2006). After wild fire, abundant orange spores of *Neurospora* species appear on fire-scorched weeds or wood. This was attributed to a unique mechanism for breaking the dormancy of the ascospores by heat and by chemicals released from burning natural objects (Pandit and Maheshwari 1996). Although *Neurospora* is not a major fungal species in nature for lignocellulosic biomass degradation, it will be a useful model organism to understand genetic and molecular mechanisms of lignocellulosic biomass degradation. The first systematic study on the topic in *Neurospora* was performed by the Glass' research group (Tian et al. 2009). Using the whole-genome microarray, they identified 769 genes that are preferentially expressed when *Neurospora* was grown in ground stem tissue of *Miscanthus*, a potential crop for biofuel production. The same authors characterized cellulase activities of mutants (from the whole-genome knockout library) that lack the expression of 16 proteins that are identified by both transcriptome and secretome analyses and found that some have higher levels of cellulase activity and some have significantly lower levels of cellulase activity in comparison to that of the wild type (Tian et al. 2009). For the last 2 years, a series of studies were reported in an attempt to elucidate a comprehensive view on regulation and mechanisms of lignocellulose degradation in *N. crassa* (Beeson et al. 2012; Bohlin et al. 2013; Coradetti et al. 2012; Dogaris et al. 2012; Fan et al. 2012; Li et al. 2012; Phillips et al. 2011; Schmoll et al. 2012;

Sun and Glass 2011; Sun et al. 2011a, 2012; Znameroski et al. 2012). With the help of the functional genomics tools, *N. crassa* could become a model fungal organism for understanding the lignocellulose degradation (Coradetti et al. 2012; Dogaris et al. 2013; Znameroski et al. 2012).

3.3.3 Soil Fungi

There is significant interest in the study of the decomposition of plant litter in soil, especially as this may be the source of many fungi of potential use in lignocellulose fermentation. Many of these studies document the decomposition rates of the liter itself and the changes in its chemistry. Others concentrate on descriptions of the organisms involved in the decomposition process and link back to the chemistry of litter by describing successions of dominant organisms based on their enzyme expression (measured in a pure culture) to resources available. Lastly, the enzymes produced by the microbial community have been studied by characterizing gross measure of enzymes in soil (Sinsabaugh 2005).

The link between changes in the fungal community during the decomposition of leaf litter has been described in terms of resource succession. Gross changes in leaf litter chemistry dictate the changes in rates of decomposition as more labile material is utilized by the initial fungal community, leaving more recalcitrant materials to be utilized by later communities with appropriate enzyme capacity (Swift et al. 1979). The communities of fungi, and presumably bacteria also, change during the decomposition of specific resources in soil. Frankland identified changes in fungal communities over time on decomposing fern petioles (Frankland 1966). Similar studies revealed changes in fungal communities over time on decaying pine needles and identified interactions between these fungi and other soil biota (Ponge 1990, 1991). Reviews of the literature on resource succession can be found in Frankland (1992, 1998). *Neurospora* occupies a specific niche in the decomposition process, occupying substrates that have recently been burned. However, our understanding of the ecology of this genus is far from complete (Lee and Dighton 2010).

Although the changes in fungal communities on decomposing leaves have been described over time as a result of resource succession, most of the hypothesized link to changes in leaf litter chemistry has been derived from gross changes in leaf chemistry (analysis of whole or multiple leaves) (Frankland 1966; Ponge 1990, 1991). More recently, we have used FTIR-ATR to investigate changes in carbohydrate chemistry of leaf material at a scale more relevant to that of the fungal mycelium (Dighton et al. 2001) and with leaf litter burning as a surrogate to decomposition (Lammers et al. 2009). Using a combination of IR and atomic force microscopy, we are now able to map the influence of individual fungal hyphae on resource surface physicochemical properties (Oberle-Kilic et al. 2013).

The ability to evaluate the functional contribution of each member of a community is important as in natural ecosystems both bacteria and fungi and possible synergistic or competitive interactions between them affect the decomposition of

plant-derived materials. However, the research on plant litter decomposition usually focuses on single species of either bacteria or fungi as the major decomposers, rather than the intra- or inter-kingdom interactions.

Each fungal species has a suite of enzymes that it can produce. These have usually been described by the expression of enzymes in culture when provided with single sources of substrate in culture conditions (e.g., clearing cellulose agar). The enzyme expression of mixtures of microbial communities has rarely been examined. Specifically we are looking for potential synergistic activities and optimization of fungal community structure to maximize rates of decomposition. In other systems, optimization of fungal communities has been shown. One example showed that it was the absolute number of ectomycorrhizal fungal species on a root system that optimized nutrient uptake by the host tree, rather than the specific fungal species comprising that assemblage (Baxter and Dighton 2001, 2005). Similarly, competition between saprotrophic phylloplane fungi and a weak plant pathogen was optimized by a mix of 4–6 species and declined with increasing numbers of saprotrophic species (Nix-Stohr et al. 2008; Stohr and Dighton 2004). Thus, it is likely that at any one time the saprotrophic microbial community on decomposing leaf material is optimized in terms of number of species and their functional capabilities. The absolute composition of the species mix may be independent of the starting community inoculated into the system.

What can we learn from the genomics studies of model fungal species, such as *Neurospora*, that will allow greater understanding of fungi in natural ecosystems? Linking molecular methods of identification and transcriptomics, Stursova et al. (2012) identified changes in fungal and bacterial communities over time in relation to their ability to decompose cellulose. Taking the leaf litter (pine needles) and upper soil from Czech forests into microcosms, they showed that the litter was dominated by fungi and soil by bacteria (Stursova et al. 2012). To each soil component (litter or soil), they added ^{13}C-labeled *Zea mays* litter to represent a cellulose resource and followed changes in fungal and bacterial communities over the next 15 days. By pyrosequencing, they identified 12,111 bacteria, 7,075 fungi, and 6,782 carbohydrolase (endocellulase) gene encoding fungi (*cbhI*). Morphologically, they identified 1,164 bacterial OUTs, 493 fungi, and 297 fungi in the *cbhI* cluster. There was a very close fit between the temporal changes in fungal community with the increased use of cellulose towards an ascomycete-dominated community. Community change was much less defined in the bacterial community. Their data suggests that the ascomycete fungi are more important for cellulose utilization than basidiomycetes. The three phases of oak litter decomposition identified by Šnajdr et al. (2011) are also fungal dominated and show changes in the suites of enzymes produced by the associated fungal communities at each stage (Šnajdr et al. 2011). During the first 4 months that represent 16% mass loss, β-glucosidase, β-xylosidase, and cellobiohydrolase enzymes dominate. Over the next 8 months, endocellulase and endoxylanase enzymes dominate and account for the 32 % mass loss. The final stage of decomposition up to 2 years, resulting in a 62 % mass loss, is largely due to ligninolytic activity.

So, what codes for these enzymes and how different are contrasting groups of fungi in their coding for these enzyme expressions? This appears to be an emerging field of interest and is especially important if we are considering taking efficient enzyme processes from one organism and inserting into other organisms with desired traits to digest lignocellulose residues to manufacture biofuels. Using an endophytic strain of the ascomycete *Chaetomium globosum*, Longoni et al. (2012) identified eight genes with potential to decompose cellulose. These were in the glucohydrolase (GH) families 5, 6, 7, 16, and 24, in common with many other ascomycetes (Longoni et al. 2012). They also found two GH 61 enzymes suggesting the possession of oxidoreductive systems in this fungal species. Similarly, Ryu et al. (2011) identified 34 likely glycoside hydrolases from the brown rot basidiomycete *Postia placenta*. However, only four of these had sequences with suspected cellulose activity (two in GH family 5, one in 10, and one in 12). In a comparison of two closely related white rot basidiomycete fungi (*Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*), Fernandez-Fuego et al. found 13 genes encoding for manganese peroxidase in *C. subvermispora*, but only 5 in *P. chrysosporium*. Additionally, *C. subvermispora* had 7 genes encoding for laccases, but *P. chrysosporium* had none (Fernandez-Fueyo et al. 2012). This explains the greater oxidoreductive capacity of *C. subvermispora* and almost exclusive lignin decomposition capacity of this species, whereas *P. chrysosporium* decomposes both lignin and cellulose. A greater understanding of the genes coding for and expression of enzyme suites in more fungal species would be of utility both to provide greater understanding of the ecological role of these fungi and to provide a resource of enzyme systems that might be exploited for commercial processes either using the fungi in which the genes occur or to transpose into other organisms that have better traits for growth in industrial processes.

Not only is it of interest to understand what enzymes fungi are capable of producing, it is also of interest to find under what conditions these enzymes are induced to be produced. The developing field of transcriptomics is likely to provide tools to allow us to determine precise conditions under which a specific fungus produces certain enzymes. However, despite literature describing details of methodology (van Elsas and Boersma 2011; Yadav et al. 2010), the use of transcriptomics in fungal ecology appears not to have been explored to a great degree. Much of the work so far has concentrated on fungal species of economic importance. For example, Nevalainen et al. (2005) investigated heterologous proteins produced by fungi for industrial use. Their aim was to improve the understanding of the process to allow gene manipulation to promote higher yield of desired end products for industrial processes (Nevalainen et al. 2005). Similarly, Andersen and Nielsen (2009) worked on the genus *Aspergillus*, where a genome of a number of species has been sequenced (Andersen and Nielsen 2009). The transcriptomics of these species has largely concentrated on the industrial uses of coded proteins or on the production of aflatoxins. A comprehensive investigation of the potential use of *Aspergillus* in polysaccharide degradation (cellulose, hemicelluloses, xylan, etc.) was conducted by Coutinho et al. (2009). They identified Carbohydrate-Active-enZyme (CAZy) family of genes coding for

polysaccharide breakdown, identifying some species where genes code for a single enzyme and other for multiple enzymes. They list 45 enzyme codes for seven substrates and 40 CAZy families with only three enzyme codes utilizing multiple substrates. Currently, the http://www.CAZy.org database lists 26 fungal species including plant pathogens, yeasts, and fungi of industrial use, but virtually no soil fungi.

Apart from transcriptomics applied to the field of mycorrhizae, few studies have looked at saprotrophic fungi in their natural environment. Eastwood et al. (2011) evaluated the evolutionary position of brown rot fungi, which utilize cellulose and hemicelluloses being derived from white rot fungi where cellulose and lignin are decomposed. Their results suggest the evolution of the ectomycorrhizal habit arose by a further reduction of polysaccharide enzyme diversity as an adaptation to an intercellular habit. Brown rot and ectomycorrhizal fungi have fewest CAZy hydrolytic enzymes, e.g., both lacking class II peroxidases.

In another study on soils of sugar maple-dominated hardwood forest across eastern North America (Kellner et al. 2010), RNA was extracted from soil samples to identify fungal genes coding for lignocellulolytic and chitinolytic enzymes. The extracted RNA was reverse transcribed, and amplified cDNAs of interest were detected at the transcript level of expression of 234 genes encoding 26 enzymes. A limitation of this study is that they looked at transcript level gene expression and not the posttranscriptional level, which means that their study does not really represent expression of the gene only the potential for expression. Thus, there is a need to look at posttranscriptional level to gain an idea of what genes are being expressed by proteomics approaches. Urich et al. (2008) applied total community RNA extraction from soil combined with reverse transcription to cDNA without PCR, followed by direct pyrosequencing. This process yielded large numbers of cDNA and rRNA tags that were deconvoluted using MEGAN software, resulting in data on diversity of organisms (taxonomic community profile based on SSU and LSU rRNA). The functional diversity of this soil community was based on broad categories of function rather than discrete enzymes (e.g., photosynthesis, phosphate metabolism, membrane transport, nitrogen metabolism). This paper seemed to have potential but did not really provide information at the individual enzyme level. These broad categories of function may be too coarse to provide useful information on the function of fungal communities in soil processes.

Other studies have looked at specific groups of fungi that have some ecological benefit. Haferburg and Kothe (2010) investigated metallomics, where they identified metal-induced changes in transcriptome, proteome, and metabolome in their search for metal resistance and metal-binding mechanisms in fungi and bacteria. They report examples of enhanced phytoremediation using helper bacteria and mycorrhizae. Lorito et al. (2010) review the large literature based on the role of *Trichoderma* as a biocontrol agent in plant pathogenesis. They review knowledge from genes to environment including transcriptome studies using ESTs (expressed sequence tags). They list a variety of potentially important enzyme functions that could be expressed by a variety of *Trichoderma* species and isolates. Their data shows a complex suite of functions, especially related to possible biocontrol of

other fungal diseases and secondary metabolites. However, most of the data are derived from pure cultures grown in a variety of conditions and thus do not represent growth in the natural environment.

In addition to the genome coding for degradative enzymes for fungal nutrient acquisition and our use of these enzymes for lignocelluloses fermentation, fungi are well known to produce a wide array of secondary metabolites. Of these, the involvement of volatile organic compounds (VOCs) in fungal–fungal and fungal–bacterial competitive interactions is an important and relatively unexplored area of fungal community dynamics. Rossouw et al. (2008) used microarrays to identify VOC production potential as secondary metabolites by wine yeasts. This was derived from fermentation studies and would be very interesting to follow with fungal–fungal interactions in the natural environment to help understand signaling processes between organisms that regulate the expression of secondary metabolite genes involved in competitive interactions.

Additional to the role of fungi in decomposition of lignocelluloses for biofuel production, the suite of enzymes that many of these Basidiomycotina possess enables them to decompose organopollutants (Šašek 2005). Potential use of these fungi for mycoremediation of areas contaminated by chlorophenols, polycyclic aromatic hydrocarbons, industrial dyes, dioxins, TNT, etc., has been proposed (Šašek 2005). The ability to insert appropriate genes to express desired enzyme suites in faster growing fungal species would be a benefit to these remediation efforts. Again, a greater understanding of the genetic regulation of enzyme expression to degrade organopollutants would allow us to better select efficient strains and optimize environmental conditions to promote degradation.

3.4 Concluding Remark

It is an exciting period of time in fungal biology. With the advancement of post-genomics tools, we may be able to test age-old ecological questions and address pressing environmental and renewable energy problems. It is our hope that more open and rigorous communications among different fields of fungal studies would enhance our understanding of both basic and applied fungal biology. The functional genomics tools developed and successfully utilized in the model organism in *Neurospora* would serve well in studying and utilizing less characterized soil fungi in diverse areas of research. The whole-genome knockout libraries of fungal species representing different functional groups (e.g., root and foliar plant pathogens, saprotrophs of contrasting taxonomic groups involved in plant litter decomposition, wood-rotting fungi) would lead to a significant progress in our understanding of soil fungal ecology.

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Chapter 4
Major Plant Pathogens of the Magnaporthaceae Family

Adriana Illana, Julio Rodriguez-Romero, and Ane Sesma

4.1 Taxonomy of the Magnaporthaceae Family

The Magnaporthaceae family (P.F. Cannon) has a complex taxonomic history. *Gaeumannomyces* and *Magnaporthe* species were grouped in the Magnaporthaceae based on the common morphology of their teleomorphs and similarities in host range (Cannon 1994). The Magnaporthaceae family is included within the class Sordariomycetes (Cannon and Kirk 2007). Traditionally, the genus *Gaeumannomyces* belonged to the order Diaporthales. Since the Magnaporthaceae family expanded to comprise fungal species that are not limited to Diaporthales fungi (Berbee 2001; Castlebury et al. 2002), enough evidence has been found to classify these fungal species at a new order level, and currently the order Magnaporthales has been proposed (Thongkantha et al. 2009). The Magnaporthaceae is a small family that comprises 17 genera and nearly a 100 species (Kirk et al. 2008; Thongkantha et al. 2009). The genera *Buergenerula*, *Ceratosphaerella*, *Clasterosphaeria*, *Clasterosporium*, *Gaeumannomyces*, *Gibellina*, *Harpophora*, *Herbampulla*, *Magnaporthe*, *Muraeriata*, *Mycoleptodiscus*, *Nakataea*, *Omnidemptus*, *Ophioceras*, *Pseudohalonectria*, *Pyricularia* and *Yukonia* belong to this family (Thongkantha et al. 2009). It is interesting to highlight that marine fungal species are included within this family such as *Buergenerula spartinae*, *Gaeumannomyces medullaris* (anamorph *Trichocladium medullare*) and *Pseudohalonectria halophila* (Jones et al. 2009).

Some *Phialophora* species have been reported as anamorphs of several *Gaeumannomyces* species including the take-all fungus *Gaeumannomyces. graminis*. The *Gaeumannomyces* species together with their *Phialophora* anamorphs and other root colonisers of non-pathogenic *Phialophora* species form the *Gaeumannomyces–Phialophora* complex (Bateman et al. 1992; Bryan et al. 1995;

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_4,
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Table 4.1 Fungal species within the genus *Gaeumannomyces*[a]

Species	Other names	Host plant	First report location	References
G. amomi		Wild ginger (*Amomum siamense* L.)	Thailand	Bussaban et al. (2001)
G. caricis	*G. eucryptus*	Marsh grass and other sedges of Cyperaceae		Walker (1980)
G. cylindrosporus	*Phialophora radicicola* var. *graminicola* ≡ *Harpophora graminicola*	Cereals and grasses		Hornby et al. (1977)
G. graminis		Cereals and grasses	Sweden	Arx and Olivier (1952) and Walker (1972)
G. incrustans		Turfgrasses	USA	Landschoot and Jackson (1989b)
G. licualae		Palm tree (*Licuala* spp.)	Australia	Frohlich and Hyde (1999)
G. medullaris	*Trichocladium medullare*	Rush (*Juncus roemerianus* L.)	USA (North Carolina)	Kohlmeyer and Volkmann-kohlmeyer (1995)
G. mirabilis	*Diaboliumbilicus mirabilis*	Palm bamboo (*Sasa veitchii* L.)	Japan	Vasilyeva (1998); (index fungorum 2: 269)
G. wongoonoo		Buffalo grass (syn. St Augustine grass, *Stenotaphrum secundatum* L.)	Australia	Wong (2002)

[a]Additional information: Freeman and Ward (2004), and Thongkantha et al. (2009)

Henson 1992; Ulrich et al. 2000). Within the genus *Gaeumannomyces*, taxonomists have identified up to nine different fungal species (Table 4.1).

Phylogenetic analyses using partial sequences of the 18S and 28S ribosomal genes of fungal isolates from different Magnaporthaceae genera suggested a monophyletic origin of this family (Thongkantha et al. 2009). However, more recent studies based on a six-gene phylogeny strongly support that both genera *Magnaporthe* and *Gaeumannomyces* are polyphyletic (Zhang et al. 2011c), meaning that they share a number of morphological signatures, but their origin probably is not from a common ancestor (convergent evolution). Therefore, the classification and evolution of *Magnaporthe*/*Gaeumannomyces* species needs further analyses by additional multigene phylogenies and whole genome comparison approaches (see Sect. 4.5).

The first original report describing the fungus *Pyricularia grisea* as the causal agent of grey leaf spot on the grass *Digitaria sanguinalis* appeared in 1880 (Saccardo 1880). A few years later, in 1892, Cavara published a report naming *Pyricularia oryzae* as the causal agent of rice blast disease (Cavara 1892). Subsequently, the name *P. oryzae* was applied for rice-infecting isolates; the isolates from other cereals and grasses kept the name *P. grisea*. However, *P. oryzae* was considered as a synonym of *P. grisea* based on morphological commonalities and interfertility between *P. oryzae* strains from rice and *P. grisea* strains from different grass hosts. Then, it was corrected to name rice-infecting isolates as *P. grisea* (Rossman et al. 1990). Concomitantly, the teleomorph of *P. grisea* was identified as *Magnaporthe grisea* (T.T. Hebert) M.E. Barr (Hebert 1971), and taxonomically it was more correct to name the sexual stage of the fungus (see further information on this subject below). As a result, scientists working on blast disease have been using four different names to refer to rice-infecting blast isolates (*P. grisea*, *P. oryzae*, *M. grisea* and *M. oryzae*).

To gain clarification on the taxonomy of *Magnaporthe* species, rice and all the other grass isolates are currently included within the *M. grisea* species complex (Couch and Kohn 2002). Globally, *M. grisea* species complex can infect a wide range of plant hosts, although one strain infects only one or few host species. Frequently one strain is susceptible only to a specific cultivar of the host (Borromeo et al. 1993; Valent and Chumley 1991). Phylogenetic analysis has inferred the presence of two monophyletic intersterile groups within the *M. grisea* species complex based on three unrelated gene sequences (actin, β-tubulin and calmodulin) and mating compatibility tests (Couch and Kohn 2002). The lineage *M. grisea* has been kept for fungal isolates associated with the host grass *Digitaria* spp. The second lineage contains rice and related fungal isolates and has been renamed as a new species, *M. oryzae*, although no morphological differences exist between isolates of these two groups.

The teleomorph is the sexual form (reproductive stage) of a fungus, while the anamorph is its asexual form. It has been a common practice to name differently the anamorph and teleomorph of a particular fungus. Based on the Article 59 of the International Code of Botanical Nomenclature, a particular fungal species with both reproductive stages, the teleomorph name takes prevalence over the

anamorph name (Hawksworth 2011). However, molecular phylogenetic approaches and whole genome sequences have revolutionised taxonomy, and this dual nomenclature rule based on morphological features is going to disappear. The rule "one fungus, one name" was approved at the Melbourne International Botanical Congress in 2011 and will be applied from January 2013 onwards (Hawksworth 2011). This has originated an important debate about the maintenance of the genus *Magnaporthe* or *Pyricularia* for the rice blast fungus among community members (http://www.magnaporthe.blogspot.com.es/).

4.2 The Take-All Fungus *Gaeumannomyces graminis*

Take-all is an extremely damaging disease of cereals and grasses caused by *G. graminis*, a homothallic soilborne fungus that colonises preferentially belowground plant tissues (Asher and Shipton 1981; Freeman and Ward 2004; Hornby 1998). Isolates of *G. graminis* are classified into four varieties based on morphological traits (ascospore size or hyphopodial structure) and pathogenicity features (host range and aggressiveness):

1. Isolates of *G. graminis* var. ***tritici*** infect mainly wheat (*Triticum aestivum* L.) but also invade barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.; Walker 1972). *G. graminis* var. *tritici* isolates can be further classified as R or N isolates based on their ability [R] or inability [N] to infect rye (Bryan et al. 1995).
2. Isolates of *G. graminis* var. ***avenae*** [*Gaeumannomyces cariceti*] infect oats (*Avena* spp.) although they can also infect rice and wheat (Dennis 1960).
3. Isolates of *G. graminis* var. ***graminis*** are less aggressive and are normally found on maize (*Zea mays* L.), rice and other grasses such as Bermuda grass (*Cynodon dactylon* L.; Arx and Olivier 1952).
4. Isolates of *G. graminis* var. ***maydis*** are found in maize and can infect *Sorghum* species (Yao et al. 1992).

It is noteworthy to mention that *G. graminis* var. *tritici* and *avenae* strains are more closely related to each other than to *G. graminis* var. *graminis* isolates (Bryan et al. 1995). Little is known about the molecular mechanisms underlying the interaction of *G. graminis* with cereal roots due to the difficulty of generating stable transformants in this fungal species, although genetic crosses and gene disruption approaches have been successfully achieved in the past with *Gaeumannomyces* strains (Bowyer et al. 1995; Frederick et al. 1999). In addition, it is a real challenge to identify and introgress take-all resistance genes in polyploid hosts such as wheat and oat.

The take-all caused by *G. graminis* var. *tritici* on wheat is one of the major agronomical problems in this crop. The most characteristic symptom is the blackening of the root due to extended necrotic lesions preceded by a complete disruption of the root architecture in severely affected crops (Fig. 4.1a). It is possible to observe black mycelia at the stem base on diseased plants. As a consequence of

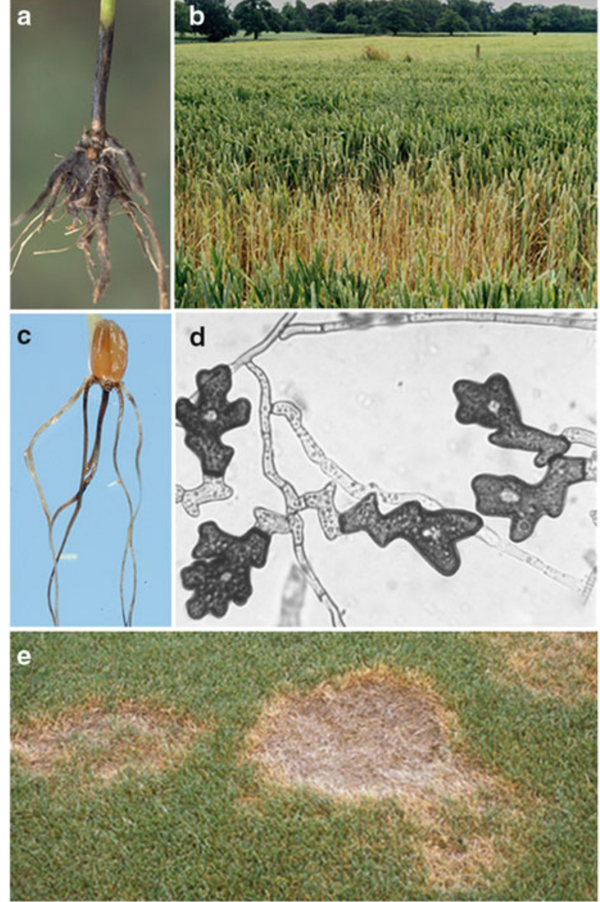

Fig. 4.1 *G. graminis var. tritici* disease symptoms. (**a**) Take-all symptoms on roots of an adult wheat plant, courtesy of Kansas State University. (**b**) Visible *white* heads and stunted plants on a wheat field infected with take-all, courtesy of Richard Gutteridge (Rothamsted Research, UK). (**c**) Necrotic lesions of a 14 days-old wheat seedling infected with *G. graminis*. (**d**) Lobed hyphopodia developed by *G. graminis* strains, with permission of Fungal Genetics and Biology. (**e**) Typical circular patches of *yellow-brown* colour of summer patch disease (*M. poae*) on turfgrass, courtesy of Dr. Lane Tredway, North Carolina State University

the collapse of the root, diseased plants tiller poorly and do not fill their heads, which become white ("whiteheads"). In the field, these symptoms are observed as round white patches (Fig. 4.1b). Disease symptoms can be present at early stages on seedlings (Fig. 4.1c). Penetration of roots by *G. graminis* is mediated by simple or lobed hyphopodia (Fig. 4.1d). Lobed hyphopodia can be melanised, but the role of the melanin in hyphopodia-mediated penetration is ambiguous in *Gaeumannomyces* species. Melanins are dark pigmented secondary metabolites produced by fungi and other organisms and play an important role in protecting these organisms against environmental stresses (Henson et al. 1999). In some phytopathogenic fungi, such as *M. oryzae*, melanin is required to keep the osmotic pressure that exerts the force for appressorium-mediated leaf penetration (De Jong et al. 1997; Howard and Valent 1996). *M. oryzae* melanin-deficient mutants are non-pathogenic. However, the involvement of melanin in pathogenicity among *Gaeumannomyces* species varies (Henson et al. 1999). Melanin-deficient mutants of *G. graminis* var. *graminis* are as virulent as the wild-type strain on rice roots.

By contrast, melanin is important for pathogenesis on *G. graminis* var. *tritici* isolates although the corresponding *Phialophora* anamorphs which are heavily melanised are non-pathogenic (Henson et al. 1999). This diverse role of melanin in root penetration and colonisation might indicate a prevalence of the mechanisms by which *G. graminis* varieties penetrate roots, i.e. turgor generation versus cell wall-degrading enzymes. Several laccases, possibly involved in the melanin biosynthesis pathway, have been biochemically characterised in *G. graminis* var. *tritici*. However, their participation in fungal virulence remains unclear due to their functional redundancy (Litvintseva and Henson 2002).

Plant cell wall represents the first barrier that any invader has to overcome to colonise the plant host. Fungal plant pathogens have developed combined strategies to cross plant cell walls. One of them is the secretion of cell wall-degrading enzymes during host invasion. Cellulose is the major polysaccharide polymer of plant cell walls (Fry 2004). It is composed of linear β(1 → 4)-linked D-glucose monomers. *G. graminis* var. *tritici* secretes endoglucanases and β-glucosidases during in vitro and *in planta* growth (Dori et al. 1995). In *G. graminis*, these enzymes have been grouped based on their acidic (4.0–5.6) and basic (≥9.3) isoelectric point. They are supposed to play an important role in cell wall degradation during *G. graminis* var. *tritici* growth on root tissues, but genetic approaches are required to confirm this hypothesis (Dori et al. 1995).

Preformed antimicrobial compounds produced by plants play an important role in plant immunity acting as first barriers to prevent pathogen attack (Field et al. 2006). The saponin avenacin is a triterpene metabolite present in the epidermal layer of oat root tips. Avenacins are a mixture of four glycosylated compounds (avenacins A1, A2, B1 and B2), and avenacin A1 is the most abundant isoform in oats (Crombie et al. 1984). Wheat roots cannot synthesise these triterpenes. While strains of *G. graminis* var. *avenae* infect oats due to their ability to synthesise avenacinase and also infect wheat, *G. graminis* var. *tritici* isolates only infect wheat roots (but not oat roots) and lack the avenacinase enzyme (Crombie et al. 1986). The fungal avenacinase detoxifies avenacin A1 into other less harmful compounds that do not affect *G. graminis* var. *avenae* growth. Taking in account that isolates of *G. graminis* var. *tritici* can infect a diploid oat species (*Avena longiglumis* L.) that lacks avenacin (Osbourn et al. 1994), a correlation exists between the presence of avenacins and the resistance of oats to non-host *G. graminis* fungal species. This was further confirmed by generating avenacinase-deficient mutants of *G. graminis* var. *avenae*, which no longer infected oat roots (Bowyer et al. 1995). Therefore, a single gene confers to *G. graminis* var. *avenae* the ability to detoxify avenacin and to control its host range.

4.3 *Magnaporthe poae*: A Root-Infecting Fungus of Turfgrasses

Magnaporthe poae affects roots and crowns of turfgrasses of the genera *Poa*, *Festuca* and *Agrostis* that are widely used in golf and other sport courses, parks and residential gardens (Landschoot and Jackson 1989a). Consequently, it is a commercially significant root-infecting fungal pathogen. The disease caused by *M. poae* is called summer patch due to the emergence of symptoms during the hot season in circular patches, which can increase up to 1 m in diameter (Fig. 4.1e). Temperature and high relative humidity favour fungal root penetration. Once inside the host, the fungus can progress through the vascular tissue to the aerial parts of the plant leading to subsequent foliar necrosis. Two new *Magnaporthe* species affecting warm-season turfgrasses have been recently described in Australia whose symptoms look similar to those produced by *M. poae* (Wong et al. 2012). These are *Magnaporthe garrettii* [P. T. W. Wong and M. L. Dickinson sp. nov.] found on couch (*Cynodon dactylon*) and *Magnaporthe griffinii* [P. T. W. Wong and A. M. Stirling sp. nov.] associated with a disease complex ("summer decline") of hybrid couch (*C. dactylon* × *C. transvaalensis*). These *Magnaporthe* species can be accurately identified in infected roots by PCR, providing a reliable method for early detection and disease management of summer patch (Zhao et al. 2012).

There is very limited information about mechanisms regulating *M. poae* infection process or plant resistance genes against summer patch disease (Tredway 2006). Serine protease activity has been observed during *M. poae* root colonisation (Sreedhar et al. 1999), suggesting an important role for this enzyme during fungal infection. Interestingly, the genome sequences of the *M. poae* strain ATCC 64411 and the Ggt isolate R3-111a-1 have been released since May 2010. The comparative analysis of the currently available genomes of Magnaporthaceae strains is still pending publication (Magnaporthe comparative Sequencing Project, Broad Institute of Harvard and MIT; http://www.broadinstitute.org/). A link may exist between their genetic intractability and their ability to colonise roots, where they have to subsist with other living organisms in the rhizosphere. Undoubtedly, the analysis of their genomes will provide many insights that will help to understand the molecular basis of ecological niche adaptation and pathogenicity in these fungal species.

4.4 Rice Blast Disease: An Important Constraint to Rice Production

Rice (*Oryza sativa* L.) is one of the most important cereal crops and staple diet of more than three billion people. Fungal blast is considered a major threat to rice crops and costs farmers a loss of nearly $5 billion a year (Skamnioti and Gurr 2009). Not surprisingly, it accounts for the world's largest fungicide market. The Japanese market alone for blast fungicides is estimated at US$400 million per year

(Skamnioti and Gurr 2009). Rice blast is caused by the fungus *Magnaporthe oryzae* (Couch and Kohn 2002), and this fungal species can also cause diseases in other staple food crops including finger millet, maize and wheat, representing a serious risk for food security globally and a significant challenge in developing countries (FAO 2009). The damage produced by blast in rice crops oscillates between 10 and 30 % every year. Under disease-conducive conditions, the fungus can destroy the entire crop (Thinlay et al. 2000). Rice blast is present in all rice-growing areas worldwide, including Western Australia where rice-growing areas were free of this disease until last year (You et al. 2012).

Rice blast is a polycyclic disease since *M. oryzae* can undergo multiple infection cycles during a rice-growing season. However, disease progression highly relies on favourable weather conditions, increasing the difficulty to effectively control blast. High humidity or long periods of rain followed by relatively warm temperatures favour spore germination and fungal penetration (Ou 1985). Wind-dispersed or water-splashed conidia are the main source of inoculum in the field (Ou 1985). However, *M. oryzae* can overwinter on alternative weed hosts and infested plant debris for almost 3 years, playing possibly an important role in the epidemiology of the disease (Harmon and Latin 2005). This fungus can form resting structures on roots and plant debris such as microsclerotia and vesicles, which can germinate even after 4 years of dormancy (Gangopadhyay and Row 1986; Sesma and Osbourn 2004) (Fig. 4.2a, b). *M. oryzae* can penetrate rice roots and spread through the vascular system to the aerial parts of the plant to produce blast disease symptoms (Fig. 4.2c, d), although the relevance of the underground infection process under field conditions is not proven yet (Besi et al. 2009; Sesma and Osbourn 2004). Domestic travellers and the transport of infected material (souvenirs made with seeds, weeds or rice straw) probably also contribute to the dissemination of the disease (You et al. 2012). PCR-based methods have been developed for detection of the fungus, offering a quick method to control the dissemination of infected material (Harmon et al. 2003).

In the field, rice blast disease symptoms are visible at any growth stage and at any part of the aerial plant tissue: leaf, collar, nodes, panicle neck and panicles (Fig. 4.2e–g). The shape, colour and size of the lesions largely depend on the rice cultivar, the age of the lesion and environmental conditions (Ou 1985). On leaves, blast lesions are eyespot shaped with white to grey colour and surrounded by a dark red-brown margin. Lesion size varies but commonly ranges between 1–1.5 cm long and 0.3–0.5 cm wide. The collar rot appears on the junction between the leaf blade and leaf sheath affecting the entire leaf. The neck rot is the most damaging symptom in the field. Typically a necrotic or rotten neck is visible at the base of the panicle often affecting the entire panicle, which becomes white and partially filled or completely unfilled. The blast symptoms in the panicle or nodes are brown or black. On roots, blast lesions show brown necrotic areas, and root architecture is maintained suggesting less aggressive damage compared to take-all symptoms caused by *G. graminis*.

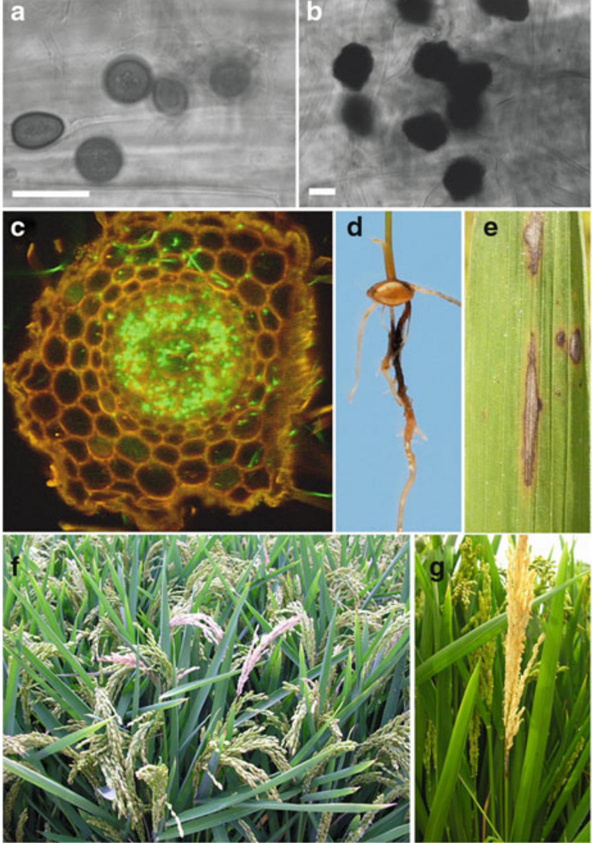

Fig. 4.2 Rice blast disease symptoms. (**a**) Fungal vesicles and (**b**) microsclerotia produced on root surfaces. (**c**) Cross section of a barley root infected with a GFP-tagged *M. oryzae* strain showing heavy colonisation of the vascular system. (**d**) Necrotic blast lesions of a 15-day-old rice seedling infected with *M. oryzae*. (**e**) Leaf blast symptoms. (**f**) Panicle blast in the field. (**g**) Neck blast symptoms. Images **f** and **g** courtesy of M. Pau Bretó (IVIA, Spain)

4.5 From Genome Sequences into Underlying Mechanisms Regulating Fungal Pathogenicity

Due to the genomic resources available for both the rice host and the fungus, the genetic tractability of *M. oryzae* and the economic relevance of blast disease, the rice–*M. oryzae* interaction has become a leading pathosystem for studying fungal pathogenicity and plant immunity in crops (Dean et al. 2012). The laboratory strain 70–15 was the first *M. oryzae* rice-infecting strain whose genome sequence was made available to the research community (Dean et al. 2005). It also represented the first genome publication of a fungal plant pathogen. The genome of *M. oryzae* is approximately 41 Mb in size (eight annotation, *Magnaporthe comparative* Sequencing Project, Broad Institute of Harvard and MIT; http://www.broadinstitute.org). Gene prediction programmes estimate the presence of 12,827 protein-coding genes, which are distributed in seven chromosomes. Optical mapping has allowed an accurate DNA alignment of the seven chromosomes.

The genome sequence of *M. oryzae* has revealed several pathogenicity-associated features. Predicted secreted proteins, which likely act as potential effectors modulating plant physiology and reducing basal host immune response, are more abundant in *M. oryzae* (~1,600) compared to *Neurospora crassa* (~800) or *Aspergillus nidulans* (~900). In addition, these non-pathogenic saprophytic fungi contain up to 10 genes encoding chitin-binding proteins, while *M. oryzae* genome has undergone an expansion on this protein family (~40 genes), indicating the complexity of chitin metabolism in *M. oryzae*. The rice blast fungus also presents an increase in seven transmembrane integral proteins, normally involved in activation of signalling pathways that help the fungus to adapt to specific external stimuli. A subgroup of these type of receptors contain CFEMs (conserved fungal-specific extracellular motif), which include an extracellular cysteine-rich EGF-like domain present exclusively in fungi (Kulkarni et al. 2005). One of the CFEM protein members, PTH11, has been shown to be involved in appressorium development and fungal virulence in *M. oryzae* (DeZwaan et al. 1999).

Different large-scale gene functional studies have been carried out since the release of *M. oryzae* genome sequence, including large-scale insertional mutagenesis (Betts et al. 2007; Jeon et al. 2007) and gene silencing (Nguyen et al. 2008). Transcriptomic approaches have also revealed global gene expression profiles during nitrogen starvation (Donofrio et al. 2006), appressorium development (Oh et al. 2008; Soanes et al. 2012) and plant infection (Mosquera et al. 2009). From the host perspective, at least 85 resistance gene loci (Pi genes), nine major QTLs defined by molecular markers and additional 350 QTLs have been identified on different rice germplasms to date (Ballini et al. 2008; Chen and Ronald 2011; Liu et al. 2010a). Furthermore, 17 resistance genes and two QTLs have been cloned since the release of the rice genome in 2002 (Table 4.2; Goff et al. 2002; Yu et al. 2002).

In 2010, and as mentioned in Sect. 4.3, two additional genomes of the Magnaporthaceae family have been made available to the scientific community (*Magnaporthe comparative* Sequencing Project, Broad Institute of Harvard and MIT; http://www.broadinstitute.org). These include the sequence drafts assemblies of the *G. graminis* var. *tritici* strain R3-111a-1 and the *M. poae* strain ATCC 64411. Although *G. graminis* var. *tritici* R3-111a-1 and *M. poae* ATCC 64411 genomes have not been assembled as well as *M. oryzae* genome, little syntenic regions exist among these three strains as shown by dot plot analysis (http://www.broadinstitute.org/annotation/genome/magnaporthe_comparative/Dotplot.html), in accordance with the polyphyletic origin of *Magnaporthe* and *Gaeumannomyces* genera found by multigene phylogeny (Zhang et al. 2011c).

More recently, the genomes of *M. oryzae* rice-infecting field isolates Y34 and P131 have been sequenced and compared against the genome reference of the laboratory strain 70–15. This genomic comparison has pointed out some relevant features of the field isolates (Xue et al. 2012). Y34 and P131 strains contain several 100 unique genes and have undergone unique DNA duplication events and expansions of pathogenicity-associated gene families. Thousands of transposon-like elements are present on the field isolates, although their genomic locations are

Table 4.2 Cloned rice blast resistance genes and associated *M. oryzae* effectors[a]

R gene	Protein domains	Cognate effector	References
Pib	NBS-LRR	–	Wang et al. (1999)
Pita	NBS-LRR	AvrPita	Bryan et al. (2000)
Pi9	NBS-LRR	–	Qu et al. (2006)
Pi2	NBS-LRR	–	Zhou et al. (2006)
Piz-t	NBS-LRR	AvrPiz-t	Zhou et al. (2006)
Pi-d2	Receptor kinase	–	Chen et al. (2006)
Pi36	NBS-LRR	–	Liu et al. (2007)
Pi37	NBS-LRR	–	Lin et al. (2007)
Pit	NBS-LRR	–	Hayashi and Yoshida (2009)
Pi5	NBS-LRR	–	Lee et al. (2009)
Pid3	NBS-LRR	–	Shang et al. (2009)
Pik-h	NBS-LRR	–	Sharma et al. (2010)
Pik-m	NBS-LRR	AvrPik/km/kp	Ashikawa et al. (2008)
Pik	Two NBS-LRR	AvrPik/km/kp	Zhai et al. (2011)
Pik-p	Two NBS-LRR	AvrPik/km/kp	Yuan et al. (2011)
Pi1	Two NBS-LRR	AvrPik/km/kp	Hua et al. (2012)
Pia	Two NBS-LRR	AvrPia	Okuyama et al. (2011)
QTLs			
Pi21	Proline rich	–	Fukuoka et al. (2009)
Pb1	NBS-LRR	–	Hayashi et al. (2010)

[a]Additional information: Ballini et al. (2008), Chen and Ronald (2011), Liu et al. (2010a) and Skamnioti and Gurr (2009)

poorly conserved among them. This suggests that transposition events might play an important role in genome variation in the rice blast fungus, which can explain the rapid adaptation of *M. oryzae* isolates to new resistant rice varieties (Kang et al. 2001; Zeigler 1998).

4.6 Evolutionary Implications of *M. oryzae* Reproduction

M. oryzae is a haploid and heterothallic ascomycetous fungus. Blast isolates with opposite mating types MAT-1.1 and MAT-1.2 (compatible strains) can conjugate and enter into an heterokaryotic stage where mycelia contain unfused nuclei (Valent et al. 1991). Subsequently, this heterokaryotic mycelium enters into a sexual cycle by fusing both nuclei. Within 3 weeks, sexual fruiting bodies or perithecia are formed. The perithecium is filled with asci, each of which contains eight ascospores (sexual spores). The dissection of ascospores is used for classical genetic studies to determine the genetic basis of phenotypic traits looking at the segregation of genetic markers (Talbot 2003; Valent and Chumley 1991; Valent et al. 1991). Blast strains isolated from finger millet (*Eleusine coracana*) or weeping lovegrass (*Eragrostis curvula*) are normally hermaphrodites and have been used to conduct early genetic studies (Valent et al. 1991). By contrast, rice

blast isolates from the same geographic location reproduce mainly asexually since the same mating type is found normally among local populations and are female sterile (Couch et al. 2005; Zeigler 1998). A few examples of fertile rice isolates have been recovered from the field such as the strain Guy11 (Leung et al. 1988; Valent et al. 1991). Transposable elements or mutations in the mating alleles are directly involved in this lack of fertility (Zeigler 1998). Heterokaryosis and parasexual cycle have been reported for rice blast field isolates (Noguchi et al. 2006). The presence in *M. oryzae* of repeat-induced point mutation (RIP)-like processes, which only occur in the sexual phase of a fungal life cycle, suggests that sexual reproduction in the rice blast fungus exists or existed in nature (Ikeda et al. 2002). Under laboratory conditions, it is relatively easy to produce sexual crosses between *M. oryzae* isolates from different grasses. This ability has been used to identify several important gene loci and to generate fertile rice-infecting laboratory strains such as 70–15.

The relevance of sexual reproduction in the field, with the advantage of increasing pathogen fitness, has been addressed in the blast fungus (Saleh et al. 2012). A direct evidence of contemporary sexual reproduction is the identification of sexual structures (perithecia) in nature. However, their visualisation is challenging since *M. oryzae* perithecia have small size and may be constrained to limited areas or time periods. Molecular tools have been developed to identify recombination events in field population samples such as linkage disequilibrium (LD) associations and diversity of molecular markers (genotyping; Arnaud-Haond et al. 2007). In populations where recombination occurs, high genotypic diversities exist, and the non-random association of alleles at two or more loci (i.e. linkage disequilibrium) is low or not significant.

As mentioned before, a similar mating type is normally found in field populations of the rice blast fungus. Strikingly, ancestral populations of *M. oryzae* from south and east of Asia, the geographical location where this fungus emerged, show clear signatures of sexual reproduction (Couch et al. 2005; Kumar et al. 1999; Saleh et al. 2012; Zeigler 1998). Molecular evidences such as genotypic richness and linkage disequilibrium data support these findings (Saleh et al. 2012). Female-fertile *M. oryzae* strains still can be recovered from these locations and can complete the sexual cycle in vitro. This is the only region in the world so far where evidences for sexual reproduction of *M. oryzae* have been found, confirming the loss of sexual reproduction outside its original location of emergence. In terms of evolution, this geographical area may represent an initial point where *M. oryzae* isolates have evolved by adaptive selection against new rice cultivars and different hosts (Saleh et al. 2012).

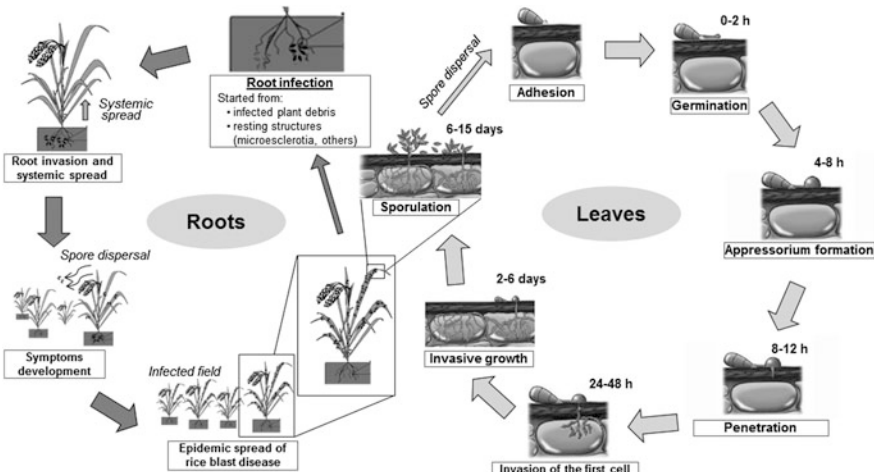

Fig. 4.3 Rice blast disease infection cycle. *Right panel*: *M. oryzae* leaf cycle modified from Ribot et al 2008. *M. oryzae* leaf infection cycle starts when a conidium lands on a leaf and attaches to the surface. Shortly after, the conidium produces a small germ tube, which differentiates into a melanised appressorium. A penetration peg formed at the base of the appressorium crosses the plant cell wall initiating fungal invasion. Invasive growth is different compared to fungal growth on surfaces. The invasive hypha moves beyond the first infected cell during a few days. Finally, conidiophores emerge and the fungus initiates sporulation between 6 and 15 days, releasing thousands of conidia to the environment. *Left panel*: *M. oryzae* root infection cycle potentially begins from infected plant debris or dormant structures present in the soil. These resting structures can germinate and penetrate into the plant roots. Fungal hypha colonises the vascular system of the root spreading systemically. The fungus moves to the upper parts of the plant producing typical blast lesions from which conidia are formed. These spores are dispersed to other plants by wind or water, propagating the disease

4.7 The *M. oryzae* Leaf Infection Process

Under high relative humidity conditions, a succession of developmental events initiates the *M. oryzae* aerial infection (Fig. 4.3; Tucker and Talbot 2001), which begins when a wind-dispersed or water-splashed conidium lands on the leaf surface. Immediately after landing, a preformed adhesive material is secreted from the conidial tip to attach itself to the highly hydrophobic surface. One hour later a short germ tube develops from the apical cell of the conidium. Within a few hours, the apex of the germ tube swells, and a specialised dome-shaped penetration structure known as appressorium is formed. The appressorium is heavily melanised and a tremendous turgor pressure is generated within this structure (De Jong et al. 1997; Howard et al. 1991). A penetration peg emerges at the base of the appressorium and crosses the plant epidermal cell by combining physical force and secretion of cell wall-degrading enzymes (Skamnioti and Gurr 2007). Subsequently, *M. oryzae* initiates a new morphogenetic programme to colonise the plant epidermal cells. Five to six days after the initial penetration of the fungus,

conidiophores emerged on the leaf surface to initiate the last step of the infection with the reproduction of the fungus.

The rice blast research community has built a large amount of information on each of the steps of the blast disease cycle. Here, a description and latest findings of the *M. oryzae* leaf infection biology follows.

4.7.1 An Extracellular Matrix Mediates Fungal Adhesion and Differentiation

Spores of *M. oryzae* get attached immediately to the highly hydrophobic leaf cuticle by secreting a preformed mucilaginous extracellular matrix (ECM). This adhesive material is passively released from the conidial apex upon hydration, meaning that there are no metabolic costs involved in this process (Hamer et al. 1988; Tucker and Talbot 2001). This attachment is required for conidial anchoring and recognition of the surface, steps that precede the subsequent infection-related development. *M. oryzae* mutants with altered ECM show reduced virulence (Ahn et al. 2004). Initial studies identified components of *M. oryzae* adhesive material such as glycoproteins and lipids which help to retain moisture, essential for appressorium-mediated penetration (Hamer et al. 1988; Howard 1997). α-Mannosyl and α-glucosyl residues are highly abundant in the ECM based on lectin labelling and protease digestions (Hamer et al. 1988; Xiao et al. 1994). Additional components of *M. oryzae* ECM have been identified by immunological techniques using antibodies against animal cell adhesion factors (collagen VI, vitronectin, fibronectin, laminin) and integrin α3 (Bae et al. 2007; Inoue et al. 2007). Particularly, collagen (as a major component), vitronectin (as cementing compound), laminin, fibronectin and integrins are present in *M. oryzae* adhesive material (Bae et al. 2007; Inoue et al. 2007). Evidences suggest that ECM components are synthesised at two different stages of the infection cycle (Inoue et al. 2007). Collagen, vitronectin and integrins seem to be formed earlier than fibronectin and laminin components.

Integrins are transmembrane glycoproteins located at the plasma membrane and act as cell surface receptors modulating the cellular response to environmental stimuli (Kim et al. 2011a; Shattil et al. 2010). Fibronectin and collagen are extracellular ligands of integrin receptors (Kim et al. 2011a; Shattil et al. 2010). Externally applied peptides containing Arg-Gly-Asp amino acids and antibodies against fibronectin reduce conidial adhesion and appressorium development indicating that these processes are modulated by integrin-like proteins in *M. oryzae*. These defects are restored by manipulating the cAMP response pathway with exogenously applied chemicals (cAMP, cutin monomers and IBMX, a cAMP phosphodiesterase inhibitor; Bae et al. 2007). These results suggest that integrin-like proteins and their cognate extracellular ligands (fibronectin, collagen) activate the cAMP-dependent pathway and possibly

other signalling pathways. This activation regulates the subsequent infection-related morphogenesis (Bae et al. 2007; Tucker and Talbot 2001). Integrins are also detected in conidial cell walls (Inoue et al. 2007), suggesting that these transmembrane receptors may play a role in the recognition of substrates by *M. oryzae* spores at earlier stages, immediately after landing.

4.7.2 Recognition of the Surface Precedes Appressorium Differentiation

Upon hydration, the first germ tube emerges, usually from the apical compartment of the conidium. If the fungus perceives that the surface is not adequate, the germ tube will arrest, blocking any further differentiation. Alternatively, it can develop a second germ tube from the opposite end of the conidium. Two germ tubes are often seen in germinating conidia on artificial substrates (Tucker et al. 2010). It is very unusual to see spores germinating from the middle compartment. The germ tube appears near the adhesion site of the spore in the apical cell and grows in direct contact with the surface of the plant for a short distance. Then, it swells and starts to change direction. This process known as "hooking" takes place before the appressorium development, and it is believed to be an important recognition step (Fig. 4.4a; Bourett and Howard 1990). During germ tube elongation, other processes such as secretion of plant cell wall-degrading enzymes, mobilisation of the metabolic reserves (trehalose) and synthesis of fungal cell wall occur (Tucker and Talbot 2001).

Concomitantly with the germination process, *M. oryzae* secretes additional compounds that contribute to the adhesion of the germ tube and perception of plant physical signals. Among them, hydrophobins have been shown to play a significant role at the early stages of fungal infection (Kim et al. 2005; Linder et al. 2005; Talbot et al. 1996). These specialised proteins are secreted at the interface between the hyphae and a hydrophobic surface and are involved in fungal development and environmental sensing (Linder et al. 2005). In *M. oryzae*, two hydrophobins play a role during infection, Mpg1 and Mhp1. Mpg1 has been widely characterised in the rice blast fungus (Beckerman and Ebbole 1996; Kershaw et al. 1998; Lau and Hamer 1996; Soanes et al. 2002; Talbot et al. 1993; Talbot et al. 1996). Mpg1 is a class I hydrophobin highly expressed during conidiogenesis, appressorium development and carbon and nitrogen starvation. The Δ*mpg1* mutants show defects in conidiation and appressoria development, and consequently are less virulent. Mhp1 is a class II hydrophobin and mutants lacking this hydrophobin show pleiotropic effects. Similarly to Mpg1, the hydrophobin Mhp1 is required for fungal morphogenesis, including appressorium development and invasive growth (Kim et al. 2005).

Cutinases and other methyl esterases are relevant enzymes secreted by fungal plant pathogens during early stages of infection (Kolattukudy 1985). Sixteen methyl esterase-encoding genes are present in *M. oryzae* genome

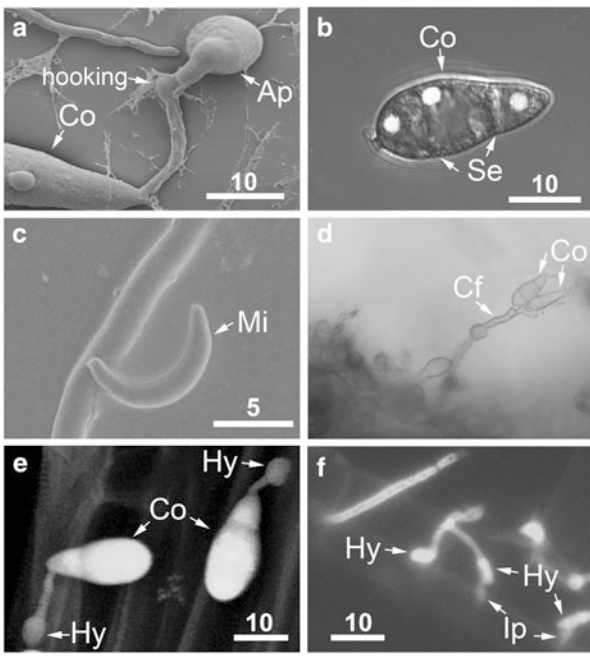

Fig. 4.4 *M. oryzae* development on artificial and root surfaces. (**a**) Scanning electron micrograph of a germinating conidium (Co) forming an appressorium (Ap) on hydrophobic coverslips. (**b**) A two-septate conidium expressing a GFP-tagged nuclear protein; septa (Se) are indicated. (**c**) Sickle-shaped microconidia (Mi). (**d**) Conidiophore (Cf)-producing conidia in the stem of a rice seedling. (**e**) Conidia on roots developing hyphopodia (Hy). (**f**) Differentiated hyphopodia from fungal hyphae producing infection pegs (Ip) to penetrate rice roots. Scale bar numbers indicate micrometres

(Dean et al. 2005), and some of them can be components of *M. oryzae* adhesive material. It is difficult to define their roles in *M. oryzae* infection biology since they may have redundant functions. Two cutinases have been characterised in the rice blast fungus. The *CUT1* gene is dispensable for *M. oryzae* plant infection (Sweigard et al. 1992). Among all the *M. oryzae* methyl esterases, *CUT2* was selected for further analysis because it is highly induced at 12 h after inoculation on barley leaves (Skamnioti and Gurr 2007). In *M. oryzae*, Cut2 acts as a surface sensor activating the cAMP/PKA and DAG/PKC signalling cascades which regulate appressorium-mediated penetration. The *Δcut2* mutants show that Cut2 is required for appressorium differentiation and full disease symptoms production, but have no defects in adhesion, indicating a specific role for a cutinase in signalling and fungal development (Skamnioti and Gurr 2007).

4.7.3 Orchestrated Cellular Processes Govern Early Stages of Plant Infection

Two important stages take place during the process of appressorium differentiation in *M. oryzae*. During the recognition phase, the apex of the germ tube begins to hook, and vesicles located in the apical area move towards the surface of the plant (Bourett and Howard 1990; Tucker and Talbot 2001). Then, the tip of the germ tube

swells and the appressorium is formed (Fig. 4.4a). This differentiation process is highly orchestrated and is activated in response to starvation stress and physical cues such as hardness and hydrophobicity (Dean 1997; Talbot et al. 1997; Tucker and Talbot 2001). Several interconnected cellular processes regulate these early stages of infection: cell cycle progression followed by cytokinesis and appressorium differentiation (Saunders et al. 2010a, b), programmed cell death (Veneault-Fourrey et al. 2006) and mobilisation of metabolic resources to generate high concentrations of glycerol for turgor-mediated penetration (Howard et al. 1991; Thines et al. 2000).

During germ tube elongation, the nucleus of the germinating cell moves towards the middle of the germ tube. Subsequently, the nucleus undergoes mitosis and one of the daughter nuclei moves towards appressoria, whereas the second nucleus returns to the conidium (Veneault-Fourrey et al. 2006). Concomitantly, storage products are transported towards the appressorium during this first nuclear division, and a septum is formed separating the appressorium from the germ tube (Saunders et al. 2010a). Then, appressorial melanisation begins. Melanin is deposited in the space between cell wall and plasma membrane to maintain appressorium integrity. High levels of glycerol derived from the degradation of stored lipids and glycogen within the appressorium build the osmotic force required for penetration of the cuticle (Thines et al. 2000). Finally, the conidium and germ tube collapse using an autophagic mechanism which is vital for pathogenicity (Talbot and Kershaw 2009; Veneault-Fourrey et al. 2006).

Appressorium morphogenesis, autophagic cell death and mobilisation of carbohydrate and lipid reserves to the appressorium are processes regulated by the mitogen-activated protein kinase (MAPK) Pmk1 pathway (Thines et al. 2000; Veneault-Fourrey et al. 2006; Xu and Hamer 1996). In eukaryotes, MAPKs are involved in the activation of cellular processes in response to environmental cues that help to adapt the cell to the exterior (Zhao et al. 2007). In *Saccharomyces cerevisiae*, five MAPK pathways exist and have been characterised in detail (Zhao et al. 2007). In *M. oryzae*, the MAPK Pmk1 (pathogenicity MAP kinase1) has been identified as the homologue of *S. cerevisiae FUS3*/*KSS1* MAPK cascades, which regulate mating and filamentous growth (Xu and Hamer 1996). The *Δpmk1* mutants are unable to produce appressorium and are non-pathogenic. However, they can recognise hydrophobic surfaces and react to exogenously applied cAMP. In *M. oryzae*, Pmk1 is also required for invasive hyphae growth (Xu and Hamer 1996). Homologues of *PMK1* are required for pathogenicity in all fungal plant pathogens (biotrophs or necrotrophs) of monocot and dicot plants studied to date, indicating that this MAPK pathway is widely conserved (Zhao et al. 2007). This pathway is under extensive analysis, and genes acting upstream and downstream of Pmk1 have been identified. These include the MAPK kinases Mst7 and Mst11 (Zhao et al. 2005); the PAK kinase Chm1 (Li et al. 2004); the Rho-GTPase MgRac1 (Chen et al. 2008); the scaffold protein Mst50 that interacts with Ras1, Ras2, Ccd42 and the Gβ subunit Mgb1 (Park et al. 2006); the membrane receptors MoMsb2 and MoSho1 (Liu et al. 2011); several transcription factors including Mst12 (Park et al. 2002), Mig1 (Mehrabi et al. 2008), MoSLF1 (Li et al. 2011) and MoMcm1

(Zhou et al. 2011); and two novel Pmk1-interacting proteins Pic1 and Pic5 (Zhang et al. 2011b).

Two other MAPK signalling pathways have been described in *M. oryzae*. The Mps1-dependent MAPK pathway is implicated in appressoria penetration and cell wall integrity (Xu et al. 1998), whereas the MAPK Osm1 is involved in the cellular response to osmotic stresses and is not required for plant infection (Dixon et al. 1999).

4.7.4 Signalling and Cytoskeletal Dynamics Regulate Fungal Plant Penetration

At the base of the appressorium, a pore ring is formed and the fungus initiates the turgor-driven penetration into plant tissues by developing a specialised hypha or penetration peg (Talbot 2003). The penetration peg enables the fungus to cross the plant cell wall and extend to the epidermal lumen of the plant. This structure is enriched in actin filaments and lacks organelles in its cytoplasm (Bourett and Howard 1992). Particularly two important signalling pathways regulate this step, the Mps1 MAPK cascade (Xu et al. 1998) and the cAMP response pathway (Xu et al. 1997).

The cAMP-dependent cascade acts cooperatively with the PMK1 pathway during *M. oryzae* plant penetration (Xu and Hamer 1996). The cAMP cascade is required for surface recognition and penetration peg emergence but not appressorium differentiation (Xu et al. 1997). The generation of glycerol and high turgor pressure within the appressorium requires a rapid degradation of lipid and glycogen reserves which is under the control of the cAMP-activated protein kinase A (PKA) pathway (Thines et al. 2000). Several key components of this signalling pathway have been studied such as the adenylate cyclase Mac1 (Choi and Dean 1997), the catalytic subunit of cAMP-dependent PKA CpkA (Xu et al. 1997), the phosphodiesterases PdeL and PdeH (Zhang et al. 2011a) and the Mac1-interating protein Cap1 (Zhou et al. 2012), which regulates the crosstalk between PMK1- and cAMP-dependent pathways through its interaction with Ras2.

Additional genetic determinants have been found to play a role in *M. oryzae* penetration including the tetraspanin *PLS1* (Clergeot et al. 2001; Lambou et al. 2008), the Pmk1-regulated genes *GAS1* and *GAS2* encoding unknown proteins conserved in filamentous fungi (Xue et al. 2002), the aminophospholipid translocase *PDE1* (Balhadere and Talbot 2001) and the isocitrate lyase gene *ICL1* of the glyoxylate cycle (Wang et al. 2003).

An actin network organised at the base of the appressorium forces the emergence of a penetration peg (Bourett and Howard 1992). In *M. oryzae*, this process is regulated by septins (Dagdas et al. 2012). Septins are highly conserved GTPases present in fungi and animals that participate in cytoskeletal-dependent cellular processes such as cytokinesis, polarity and secretion (Gladfelter 2006; Mostowy and Cossart 2012).

Septins also act as diffusion barriers. *M. oryzae* genome contain five septin genes, four of which are core septins present in budding yeast. *M. oryzae* septins form a dynamic septin ring that contributes to the formation of a toroidal filamentous actin network surrounding the appressorial pore, where the penetration peg differentiates (Dagdas et al. 2012).

4.7.5 *Insights into* M. oryzae *Invasive Growth*

Within the host cell, the fungus develops several types of biotrophic invasive hyphae (IH; Kankanala et al. 2007), which contain distinct morphological features compared to the filamentous hyphae produced on the leaf surface or in vitro. Soon after the *M. oryzae* penetration peg has crossed the epidermal cell wall, it differentiates into a short and thin filamentous hypha known as primary IH. This primary IH precedes the formation of a thicker intracellular pseudohypha called bulbous IH. The bulbous IH grows within the cytoplasm and moves beyond the first invaded cell by crossing with constricted infection pegs at regions of the plasma membrane where plasmodesmata aggregate, also known as pit fields (Bell and Oparka 2011). Thereafter, the bulbous IH differentiates into filamentous IH in the new invaded cell and subsequent fungal invasion continues into neighbouring cells (Kankanala et al. 2007). Importantly, bulbous and filamentous IH are not in direct contact with the plant cell cytoplasm since a plant-derived plasma membrane called extra-invasive hyphal membrane (EIHM) surrounds them. There is no well-established matrix between IH and EIHM, and IH grows in close contact with the EIHM (Kankanala et al. 2007). Secreted fungal proteins and other compounds are retained inside this space such as Slp1 and Bas4 effectors or can be translocated into the plant cytoplasm as it is the case for Pwl2 (described later). An additional morphological feature of *M. oryzae* invasive growth is the formation of biotrophic interfacial complexes (BICs) where effector proteins accumulate (Khang et al. 2010). When *M. oryzae* penetrates the first cell, a BIC is formed at the tip of the first bulbous IH, and is left behind, remaining as a discrete structure while the bulbous IH continues growing. New BICs are observed at the tip of each IH growing inside the plant cell. Five to six days after the initial fungal infection, conidiophores start to emerge in the leaf surface, and spores are produced massively during 2 weeks.

The elucidation of the molecular mechanisms involved in *M. oryzae* invasive growth has been largely overlooked because many of the mutants characterised in this organism are penetration defective. There is an extensive coupling between penetration and invasive growth processes since cell wall degradation and mechanical pressure are also involved during fungal growth inside the host cells (Heath et al. 1992; Xu et al. 1997). As an example, *Δmst12* fails to penetrate onion epidermal cells and to infect wounded leaves although it differentiates melanised appressoria, indicating that Mst12 is required for both penetration peg formation and invasive growth differentiation (Park et al. 2002). Very few genes have been

found to play specifically a critical role during *M. oryzae* plant invasion. *MIG1* is involved in the late stages of *M. oryzae* infection since the *Δmig1* mutants form normal appressoria, penetrate host cells and develop primary IH but fail to infect wounded leaves. Mig1 is one of the two MADS-box transcription factors present in *M. oryzae* and a downstream target of the MAPK Mps1 (Mehrabi et al. 2008). The *MIR1* gene specifically expressed in *M. oryzae* IH encodes a protein of unknown function, which is present only in the *M. grisea* species complex. Despite the fact that *MIR1* expression is exclusively found in IH, *Δmir1* mutants have no defects in appressorial penetration and are fully pathogenic (Li et al. 2007).

4.7.6 Fungal Metabolism and Plant Infection

M. oryzae has to adapt to the changing nutritional environment during host invasion, and consequently metabolism plays an essential role during *M. oryzae* invasive growth. *M. oryzae* is considered a hemibiotrophic fungus based on its nutritional mode during host invasion. However, genes regulating the switch in life style and acquisition of nutrients during plant infection are largely unknown (Fernandez and Wilson 2012; Kankanala et al. 2007). The duration of the biotrophic versus the necrotrophic phase in *M. oryzae* is also unknown. During early stages of rice colonisation, *M. oryzae* grows and fulfils its nutritional needs from the plant tissue without killing the host cells due to its ability to manipulate rice physiology as many other biotrophs do (Mendgen and Hahn 2002; Mengiste 2012). During this biotrophic stage, limited amounts of cell wall-degrading enzymes are produced and toxin production is absent according to a biotrophic life style. By contrast, extensive degradation of plant cell walls is observed at later time points of infection and in heavily invaded tissues, both stages associated with the necrotrophic phase of the fungus (Kankanala et al. 2007; Rodrigues et al. 2003). Typically, necrotrophs produce phytotoxic compounds and cell wall-degrading enzymes to kill the cells and cause leakage of nutrients (Mengiste 2012). Plant cell walls nearby *M. oryzae* hyphae show strong enzymatic digestion, correlating with *M. oryzae* necrotrophic phase (Kankanala et al. 2007).

Possibly one of the cues that trigger the switch from biotrophic to necrotrophic hyphae in *M. oryzae* is the lack of carbon sources within the host cell. It is known that nutrient starvation also can act as an environmental cue for infection-related differentiation (Talbot et al. 1997). *M. oryzae* has to limit the acquisition of nutrients during its biotrophic phase to maintain host cell integrity. Consequently, the use of nutrients must be highly regulated during *M. oryzae* biotrophic growth in order to respond appropriately to nutrient availability. Several interconnected pathways regulate *M. oryzae* growth in response to nutrients during plant invasion. These include the target of rapamycin (TOR) signalling cascade, carbon catabolite repression (CCR), nitrogen metabolite repression (NMR) and the integration of carbon and nitrogen metabolism by trehalose-6-phosphate synthase 1 (Tps1).

The TOR signalling cascade is an intracellular regulatory network used by eukaryotic cells to regulate growth according to nutrient availability. The 14-3-3 proteins are involved in key cellular processes and integrate environmental cues through the regulation of signalling pathways, including TOR. The TOR signalling pathway is regulated by the RNA-binding protein Rbp35 (Franceschetti et al. 2011). Rbp35 is a component of the polyadenylation machinery, and it is required for alternative 3' end processing of pre-mRNAs. One of the RBP35 targets is the 14-3-3 pre-mRNA, and this could explain the defects that Δ*rbp35* shows on TOR signalling and plant infection.

NMR is a highly regulated process in which preferred nitrogen sources, such as ammonia, glutamine and glutamate, are used preferentially. Ammonia is the preferred nitrogen source for *M. oryzae*. The NMR in *M. oryzae* occurs through the transcriptional activator Nut1, the *M. oryzae* AreA/Nit2 orthologue (Froeliger and Carpenter 1996). The expression of a large number of genes encoding enzymes that are involved in the utilisation of various secondary nitrogen sources—nitrate, purines or amino acids—is subject to nitrogen metabolic repression and is positively regulated by Nut1. The Δ*nut1* mutant can grow on ammonia, which does not require an active Nut1, but Δ*nut1* is unable to grow on certain alternative nitrogen sources such as nitrate. *M. oryzae* mutants in genes involved in nitrate assimilation and whose expression is regulated by Nut1 such as *NIA1* and *NIR1* are fully pathogenic on rice leaves (Lau and Hamer 1996; Wilson et al. 2010). This suggests that NMR is not involved in *M. oryzae* leaf colonisation and consequently the fungus can assimilate preferred sources of nitrogen (ammonia, glutamine or glutamate) from aerial host tissues. However, genes involved in response to nitrogen availability are important for infection. Two *M. oryzae* nitrogen-regulatory genes of unknown identity, *NPR1* and *NPR2*, are required for growth on a wide range of secondary nitrogen sources, including nitrate, and do not develop lesions on barley (Lau and Hamer 1996). Therefore, nitrate is not required for *M. oryzae* leaf infection, but secondary nitrogen sources assimilated via *NPR1* or *NPR2* are necessary for development of full disease symptoms. Additionally, several studies have shown that nitrogen-limiting conditions result in the expression of genes required for fungal pathogenicity such as the genes encoding the hydrophobin *MPG1* and the vacuolar subtilisin-like protease *SPM1* (Donofrio et al. 2006; Saitoh et al. 2009; Soanes et al. 2002). We require further studies to understand the molecular mechanisms underlying NMR and their involvement in nitrogen assimilation during *M. oryzae* plant infection.

CCR is a genetic mechanism that ensures the preferential use of glucose over other, less-preferred carbon sources, and it is also present in *M. oryzae* (Fernandez et al. 2012). *M. oryzae* has the ability to use a wide range of mono- and disaccharides as sole carbon source but has a strong preference for glucose (Fernandez and Wilson 2012; Tanzer et al. 2003; Wilson et al. 2007). In *A. nidulans*, CCR is mediated at DNA level by the global transcriptional repressor CreA. A putative orthologue of CreA (MGG_ 11201) is present in *M. oryzae*, and its role in fungal pathogenicity has yet to be elucidated.

An interesting interconnection of NMR and CCR is mediated by the sugar sensor trehalose-6-phosphate synthase (Tps1) and trehalose-6-phosphate (Fernandez et al. 2012). Tps1 is one of the three mediators of CCR identified in *M. oryzae*. The other two mediators are the Nmr1/2/3 inhibitor proteins and Mdt1, a multidrug and toxin extrusion (MATE)–family pump. Tps1 is a metabolic enzyme that synthesises trehalose-6-phosphate (T6P, a trehalose intermediate) from UDP-glucose and glucose-6-phosphate (G6P). Tps1 has two roles, as a biosynthetic enzyme and as signalling component of G6P. The sensing of G6P by Tps1 results in activation of the activity of the enzyme glucose-6-phosphate dehydrogenase (G6PDH), which converts NADP to NADPH using G6P in the pentose phosphate pathway. Therefore, Tps1 controls intracellular levels of NADPH (depending on the concentration of G6P) and subsequent activation of NADPH-dependent signalling cascades that interconnect carbon and nitrogen metabolism. When NADPH levels increase in a Tps1-dependent manner, three NADP-dependent inhibitor proteins (Nmr1 to Nmr3) are inactivated. As a result of inactivation of Nmr proteins, at least three GATA transcription factors become active, one of which is the white collar-2 homologue involved in light sensing (Pas1). The other GATA factor is essential for appressorium formation (Asd4), and the third GATA factor is Nut1 (Wilson et al. 2010). The modulation of GATA factor activity in the NADPH-dependent signalling pathway results in Tps1-dependent expression of at least three known virulence factors: the melanin enzyme Alb1, the seven transmembrane receptor Pth11 and the hydrophobin Mpg1 (Wilson et al. 2007). Accordingly, Δ*tps1* mutants are non-pathogenic. Tps1 regulation of Nut1 results in similar but not identical growth phenotype of Δ*tps1* and Δ*nut1* strains on a wide range of nitrogen sources. An additional regulator of the CCR signal transduction pathway in *M. oryzae* has been identified during a forward suppressor screening in Δ*nut1* background (Fernandez et al. 2012). Mdt1 is a member of the MATE protein family required for sporulation and plant infection but not appressorium differentiation. Mdt1 regulates carbon metabolism via extrusion of citrate during infection and growth contributing to *M. oryzae in planta* nutrient adaptation.

In summary, NADPH signalling, CCR, NMR and TOR are mechanisms by which *M. oryzae* can sense and adapt its metabolic status to nutrient availability during *in planta* growth. Future research will determine the interplay among these regulatory pathways that play a pivotal role in the establishment of plant disease.

4.7.7 Secretion Systems: Effectors, Toxins and ABC Transporters

Plant recognition of conserved microbial features (pathogen- or microbial-associated molecular patterns, PAMPs or MAMPs) such as chitin or flagellin (Howard et al. 1991) is mediated by pattern recognition receptors (PRRs; Zipfel 2008). During the coevolution of plants and associated pathogens, plants have developed two levels of

immune responses (Jones and Dangl 2006), the PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). In general, PAMPs are conserved among species of pathogens and play an essential role in pathogenicity. Therefore, PTI represents the first level of immune response in a host. The second type of plant innate immunity, the ETI, is activated upon recognition of highly diverse molecules secreted by the pathogens known as effectors. Fungal effectors play an essential role during invasion (Hogenhout et al. 2009; Stergiopoulos and de Wit 2009). Successful pathogens have managed to produce effectors that overcome PTI. Conversely, some plant resistance genes have evolved to recognise such type of effectors blocking their effect (ETI). Then, plant pathogens no longer can infect their host and become non-pathogenic or avirulent (Jones and Dangl 2006).

M. oryzae contains ~1,600 predicted secreted proteins that may play a role during rice infection (Dean et al. 2005; Soanes et al. 2008). It is not easy to assign a role in pathogenicity to an effector protein by gene disruption due to the large amount of secreted proteins, possibly with functional redundancy, present in *M. oryzae* (Saitoh et al. 2012). Two effector proteins with virulence functions have been characterised in *M. oryzae*, MC69 (Saitoh et al. 2012) and Slp1 (Mentlak et al. 2012). MC69 is a single secreted protein that is indispensable for virulence in fungi pathogenic on both monocots and dicots. When MC69 is absent, *M. oryzae* pathogenicity is severely reduced after penetration into the host cells. However, there are no clear evidences supporting how MC69 contributes to pathogenicity or virulence. The Secreted LysM Protein1 (Slp1) has two LysM domains involved in carbohydrate recognition and is secreted into apoplastic space during initial invasive growth in *M. oryzae*. This protein is only expressed during the biotrophic phase of *M. oryzae* (Mentlak et al. 2012). Slp1 can be glycosylated and can form oligomers (Mentlak et al. 2012). The Δ*slp1* mutants show reduced disease symptoms due to their defects in invasive growth (Mentlak et al. 2012). The Slp1 effector competes with the plant chitin receptor CEBiP to attenuate the rice immune response, the PTI, activated by the presence of *M. oryzae* chitin oligosaccharides.

To date, the majority of the effectors identified in *M. oryzae* act as avirulence (AVR) proteins triggering effector-mediated cell death (or ETI) and blocking subsequent pathogen invasion. However, their mode of action is still largely unknown at the molecular level. De novo sequencing of the Japanese rice isolate Ina168 genome and its comparison with the reference genome 70–15 has allowed the identification of a genomic region present only in Ina168 that contained three *AVR* genes (*AVR-Pia*, *AVR-Pii* and *AVR- Pik/km/kp*) (Table 4.2; Yoshida et al. 2009). An additional effector gene identified by map-based cloning is *AVRPiz*-t (Li et al. 2009). Knockout mutants in all these genes fail to show virulence phenotypes except in their specific cultivars containing the matching resistance genes. AvrPiz-t is able to suppress BAX-mediated programmed cell death in tobacco leaves in transient expression experiments, providing evidence that this effector may have a role in suppression of plant immunity. An interesting case of a protein with AVR effector function is *M. oryzae* Ace1. Ace1 is a polyketide synthase–nonribosomal peptide synthetase (PKS-NRPS) located within a gene cluster involved in the biosynthesis of secondary metabolite(s). The

metabolite synthesised by the *ACE1* gene product represents the only secondary metabolite found in *M. oryzae* so far with an avirulence role (Collemare et al. 2008; Fudal et al. 2007). *M. oryzae* isolates containing the *ACE1* gene are unable to infect rice cultivars containing the resistance gene Pi33 (Berruyer et al. 2003). The *ACE1* gene is exclusively expressed *in planta*, making it difficult to identify the Ace1-dependent natural product. *ACE1* expression is tightly coupled to the onset of appressorium-mediated penetration of the host cuticle.

Effectors are also involved in determining *M. oryzae* host species specificity. The *M. oryzae* AVR effector Pwl2 (pathogenicity towards weeping lovegrass 2) prevents *M. oryzae* isolates from infecting weeping lovegrass (Sweigard et al. 1995). The *PWL* gene family consists of four genes *PWL1*, *PWL2*, *PWL3* and *PWL4*. Pwl1 is a functional AVR effector and has 78 % nucleotide identity with Pwl2. Pwl2 accumulates in the BICs, and this property correlates with its translocation across the plasma membrane into the rice cytoplasm. There are no evidences of avirulence roles for Pwl3 (63 % nucleotide identity) and Pwl4 (65 % nucleotide identity; Kang et al. 1995). Additional *AVR* genes identified in *M. oryzae* field isolates are *AVR1-CO39*, which is broadly present in *M. oryzae* populations adapted to other host species, and *AVR-Pita1* (Valent et al. 1991). *AVR-Pita1* is a subtelomeric effector gene which has been extensively studied to understand *AVR* gene evolution among field isolates in order to generate valuable information for the deployment of resistance genes in field crops (Chuma et al. 2011; Jia et al. 2000).

Four additional biotrophy-associated secreted (Bas1 to Bas4) protein effectors are expressed during biotrophic invasion but not in vitro (Khang et al. 2010). Bas1 is translocated into the rice cell cytoplasm and shows preferential accumulation in BICs, like Pwl2. *M. oryzae* translocated effectors moved ahead of the fungus and can be seen in the absence of invasive hyphae within the cells, suggesting that these effectors prepare host cells prior to fungal invasion (Khang et al. 2010). It is not clear how *M. oryzae* delivers effector proteins during its biotrophic phase into the host cells. The *MgAPT2*-dependent polarised exocytotic processes might contribute to the secretion of effectors during *M. oryzae* plant colonisation (Gilbert et al. 2006). Bas2 and Bas3 are found in BICs, but they also localise in cell walls of invasive hyphae. Bas4 is a potential matrix protein that preferentially accumulates between the EIHM and the *M. oryzae* cell wall. The knockout mutants in the *BAS* genes show no particular phenotype, indicating the functional redundancy of the fungal secretome. Some of these Bas proteins might be involved in altering plant components required for biotrophic invasion, but no clear evidences have been reported (Khang et al. 2010).

In addition to effector proteins, *M. oryzae* also secretes phytotoxins although this is a largely unexplored area. Pyriculol, tenuazonic acid and pyrichalasin H have been isolated from culture filtrates of *M. grisea* isolates (Tsurushima et al. 2005). Pyriculol induces necrosis and it is widely distributed among *Magnaporthe* species. Tenuazonic acid is also present in *Alternaria* species. Pyrichalasin H is a cytochalasin that prevents polymerisation of actin filaments and is able to inhibit rice seed development although it is not required for leaf disease symptoms. Pyrichalasin H

is exclusively produced by blast isolates that infect *Digitaria* plants, and possibly it represents a host specific toxin (Tsurushima et al. 2005). Lately, the Mag-toxin has been purified from *M. oryzae* isolates infecting *Avena* species. Mag-toxin is a derivative of linoleic acid and only causes chlorosis in the presence of light. This toxin is able to induce mitochondria-associated ROS production and cell death (Tsurushima et al. 2010).

Plants secrete toxic compounds to defend themselves from pathogens. The ATP-binding cassette (ABC) transporters play an essential role in fungal survival allowing to efflux plant antimicrobial substances to the cell exterior (Coleman and Mylonakis 2009). *M. oryzae* has about 50 ABC transporters (Coleman and Mylonakis 2009). Four ABC transporters have been characterised in *M. oryzae*. Abc1, Abc3 and Abc4 are required for pathogenicity but are dispensable for appressorium differentiation (Gupta and Chattoo 2008; Sun et al. 2006; Urban et al. 1999). Mutants in these genes differentiate normal appressoria and are either unable to penetrate or die shortly after penetrating the host cell. The best characterised ABC transporter is Abc3, which localises in the plasma membrane of appressoria (Sun et al. 2006), and pumps out a plant-derived steroidal glycoside (Patkar et al. 2012).

4.7.8 Conidiation and Light Regulation

The sporulation process is an essential step for fungal reproduction and dispersal and influences largely the disease progression in the field. *M. oryzae* can produce two types of spores. Some *M. oryzae/grisea* isolates produce single-celled microconidia (Chuma et al. 2009; Kato et al. 1994) (Fig. 4.4b). Microconidia have thin cell walls and lack nucleoli. They have been identified in other fungi—*N. crassa*, *Botrytis cinerea* or *Podospora anserina*—where they play a role as spermatia during sexual reproduction (Fukumori et al. 2004). Mature microconidia show lower metabolic activity compared to germ tubes, indicating that they may be quiescent or dormant. The *M. oryzae* MADS-box transcription factor MoMcm1 regulates microconidia production and is also involved in male fertility, supporting the role of microconidia as spermatia during the sexual cycle of *M. oryzae* (Zhou et al. 2011).

Macroconidia (also named conidia or asexual spores) represent the main dispersal forms of the blast fungus. *M. oryzae* conidia are pyriform (pear shaped) and bisepted (occasionally 1 or 3 septa can be seen). These two septa generate three distinct cellular compartments in the conidium, each of them enclosing a nucleus (Fig. 4.4c). Conidia size ranges between 19–27 μm long and 8–10 μm wide. Normally, conidia present a basal appendage at the point of attachment to the conidiophore. Conidiophores are specialised hyphae up to 130 × 3–4 μm in size, and conidia are formed in their apex (Fig. 4.4d). A mature *M. oryzae* conidiophore rarely branches and can form between three and five conidia sympodially arranged.

Conidiophores emerge to the plant cell surface and release conidia into the environment.

Molecular mechanisms governing conidiation have been characterised in exquisite detail for the model organisms *A. nidulans* and *N. crassa* (Etxebeste et al. 2010; Park and Yu 2012). Conidiation-defective genes and genetic loci have also been identified in the rice blast fungus such as the *CON* mutants (Shi et al. 1998), *ACR1* (Lau and Hamer 1998), *COS1* (Zhou et al. 2009), *SMO* (Hamer et al. 1989), *CDC15* (Goh et al. 2011) and *COM1* (Yang et al. 2010). A genome-wide expression profile using spores from the rice isolate KJ201 has identified several hundred genes to be up- or downregulated during *M. oryzae* conidiation, approximately 4.5 % of its total gene content (Kim and Lee 2012). A further comparative transcriptome analysis between the wild-type strain and the Δ*mohox2* mutant under sporulation conditions has identified a subset of conidiation-related genes regulated by the homeobox transcription factor MoHox2/Htf1 (Kim et al. 2009; Liu et al. 2010b). Δ*Mohox2* mutants fail to produce conidia indicating that this transcriptional regulator plays an essential role in *M. oryzae* conidiation process. Not surprisingly, expression of *M. oryzae* genes *MoCON6*, *ACR1*, *MoBRLA* and *MoFLBC* is significantly upregulated during conidiation in the wild type but not in Δ*mohox2*. These genes are also highly expressed during sporulation in other fungal species (Adams et al. 1988; Etxebeste et al. 2010; Kwon et al. 2010; Springer and Yanofsky 1992). By contrast, the expression of *M. oryzae MoFLBA* and *MoVOSA* (the *A. nidulans flbA and vosA* orthologues, respectively) is significantly downregulated or unaltered in the wild type while is highly upregulated during conidiation in *A. nidulans*. The *M. oryzae* Δ*vosA* mutant has no defects in conidiation although the *A. nidulans* VosA is a key regulator of the sporulation process. This may suggest that gene pathways regulating conidiation differ between fungal species because they derive from new mechanisms of gene regulation, rather than biochemical function. Further investigation is necessary to define the genetic pathway and molecular mechanisms controlling conidiation in *M. oryzae*.

The light is an environmental factor that influences several biological processes in *M. oryzae* such as conidiation. It is necessary to grow *M. oryzae* under light/dark conditions to get good sporulation rates (Lee et al. 2006). Asexual development and light regulation are interconnected processes in *A. nidulans* and *N. crassa* (Olmedo et al. 2010a, b; Ruger-Herreros et al. 2011). The light during asexual development affects mainly aerial hyphae and conidiophore differentiation. Conidiation in *M. oryzae* is suppressed by blue light during light/dark cycling and the release of conidia is controlled by both blue and red light (Lee et al. 2006). Therefore, *M. oryzae* senses the light-to-dark transition, and this environmental cue triggers asexual differentiation and spore release. It is clear that environmental light also influences *M. oryzae* interaction with rice. It seems that a dark phase applied immediately after pathogen–host contact plays a critical role for disease development (Kim et al. 2011b). Significant light-dependent disease suppression is observed in rice plants infected with *M. oryzae* when plants are exposed to light (instead of darkness) directly after inoculation (Kim et al. 2011b). In nature, it is difficult to establish the contribution of a particular environmental factor to disease

progression since environmental factors are interdependent and can affect the host physiology (plant), the pathogen physiology (fungus) and/or the interaction between both organisms. A partial "blind" strain of *M. oryzae* required for darkness sensing (a knockout strain in *MgWC*-1, the blue light photoreceptor gene) has allowed to dissect the effect of light in the fungus during disease development. MgWc-1 is required for light-dependent disease suppression during the dark phase (disease-conducive light condition) after pathogen–host contact. In other words, a full disease progression requires a light/dark cycle after pathogen–host contact and light-to-dark transition sensed by photoreceptors. However, appressorium differentiation and penetration is not regulated by light, and therefore, light does not affect early stages of *M. oryzae* plant infection. Plants are subject to an overall greater pathogen challenge during the night. Possibly the fungus recognises darkness to mobilise fungal effectors (and also possibly metabolic reserves) during invasive growth, as has been suggested for *Cryptococcus neoformans*, as a mechanism to avoid the light-regulated increased defence responses in plants (Griebel and Zeier 2008; Idnurm and Heitman 2005). Light-to-dark transitions must be taken in account to understand the crosstalk between plant and associated fungal pathogens, considering that both organisms have an active circadian clock.

4.8 The Dark Phase of Blast: *M. oryzae* Root Infection Biology

Similar to its close relatives, *M. oryzae* infects roots (under laboratory conditions) and undertakes a set of developmental programmes typical of root-infecting pathogens (Sesma and Osbourn 2004; Tucker et al. 2010). Several key differences have been found between the mode of penetration of leaves and roots. In contrast to the melanised appressoria observed on leaves, *M. oryzae* produces hyphal swellings to penetrate roots, resembling the simple hyphopodium seen in root-infecting fungi of the *G. graminis–Phialophora* complex (Fig. 4.4e). *M. oryzae* hyphopodia are not melanised and *M. oryzae* melanin-deficient mutants are able to produce hyphopodia and infect roots (Sesma and Osbourn 2004). The PKA regulates the high turgor pressure within appressoria generated by the degradation of lipid and glycogen reserves (Thines et al. 2000). The *M. oryzae Δcpka* mutant produces hyphopodia and penetrates roots, indicating that root colonisation is not dependent on *CPKA* (Sesma and Osbourn 2004). Consequently, *M. oryzae* penetrates the epidermal root cells through a melanin-independent mechanism and the mechanical entry of the hard leaf surface by osmotic force is not operational during hyphopodia-mediated root penetration. From the host perspective, defence-related gene transcripts of rice showed a different temporal induction pattern during *M. oryzae* infection of leaves or roots (Marcel et al. 2010), which correlate with the different invasion mechanisms that the rice blast fungus undertakes for colonisation of leaves and roots.

Pre-invasive hyphae (pre-IH) are another type of fungal development observed on root surfaces that also mediates direct penetration of epidermal root cells (Tucker et al. 2010). *M. oryzae* pre-IH is developed from hyphopodia or germ tubes and penetrates roots directly. The pre-IH can be followed by differential labelling with concanavalin A and wheat germ agglutinin, which indicates that cell wall changes accompanied to this morphogenetic programme. Artificial surfaces such as hydrophilic polystyrene (PHIL-PS) can induce hyphopodia-like structures and pre-IH. The mutant *Δpmk1* is non-pathogenic on roots (Dufresne and Osbourn 2001), and this mutant is unable to develop pre-IH on roots and PHIL-PS. Consequently, this fungal differentiation is regulated by the MAPK Pmk1 cascade. Other structures typical of root-infecting fungi seen during *M. oryzae* root colonisation include microsclerotia and resting structures such as vesicles and swollen cells (Gangopadhyay and Row 1986; Lee et al. 2000; Sesma and Osbourn 2004).

Several lines of evidence have led to the hypothesis that the hyphopodium is an intermediate step before appressorium penetration. It is possible that the primitive hyphopodia evolved by acquisition of melanin and generation of high turgor pressure into a more sophisticated penetration structure, the appressorium (Tucker et al. 2010). The screening of *M. oryzae* insertional library of 2,885T-DNA transformants looking for altered pre-IH differentiation mutants on PHIL-PS has identified 20 transformants that show reduced virulence or are non-pathogenic on leaves and/or roots (Tucker et al. 2010). Further analysis of these mutants has revealed that appressorium, hyphopodium and pre-IH formation are highly coupled developmental processes, and very few mutants show an organ-specific involvement for infection (Tucker et al. 2010). This indicates that a significant set of common genes are necessary for fungal infection on both plant organs. Out of the 20 mutants, M1373 shows a root-specific infection-deficient phenotype (Table 4.3). This mutant lacks the *M. oryzae* orthologue of exportin-5/Msn5p (EXP5). The defects of the *Δexp5* mutant on disease symptoms production are more evident on roots than on leaves. *M. oryzae* EXP5 presents a steady-state nuclear localisation under all the conditions tested. *Δexp5* mutants show a reduction in conidia production (ca. 40 times lower) and altered preinvasive growth on PHIL-PS. The perimeters of the leaf lesions produced by *Δexp5* are smaller, which suggests deficiencies in invasive growth. Pathogenesis-related proteins and/or RNAs transported by this nucleocytoplasmic receptor play a crucial role during *M. oryzae* infection-associated development.

Exp5 may be involved in the nucleocytoplasmic transport of proteins implicated in nitrogen assimilation. Differences have been found in the role played by nitrogen-related genes during *M. oryzae* leaf and root colonisation. The assimilation of nitrogen by *M. oryzae* from underground plant tissues is regulated by the global nitrogen regulator Nut1 (Froeliger and Carpenter 1996). The *Δnut1* mutant is non-pathogenic on roots but infects leaves as well as the wild-type strain (Dufresne and Osbourn 2001). Consequently, *M. oryzae* absorbs nitrogen from less preferred sources in root tissues, and therefore, the NMR plays a crucial role during root infection. The mutants *Δnpr1* and *Δnpr2* are non-pathogenic on leaves and show opposite phenotypes on roots (Table 4.3), representing an additional evidence of the different roles that nitrogen-related genes play during *M. oryzae* colonisation of leaves and roots.

Table 4.3 Organ-specific and general pathogenicity genes regulate *M. oryzae* plant colonisation

M. oryzae strains	Targeted gene function	APP[a]	Leaf symptom[b]	HY[c]	Root symptom	References
Wild type		Yes	+++	Yes	+++	
Root specific						
Δmgfow1	Mitochondrial respiration	Yes	+++	+	+/++	Sesma and Osbourn (2004)
Δexp5	Karyopherin	Yes	++	ND	–	Tucker et al. (2010)
Δnut1	Nitrogen global regulator	Yes	+++	+	–	Dufresne and Osbourn (2001) and Froeliger and Carpenter (1996)
General						
Δmagb	Gα subunit	Yes	–	ND	–	Fang and Dean (2000)
Δabc1	ABC transporter	Yes	–	ND	–	Dufresne and Osbourn (2001) and Urban et al. (1999)
Δmgapt2	P-type ATPase	No	–	ND	–	Gilbert et al. (2006)
Δnpr2	Nitrogen metabolism	Yes	–	ND	–	Dufresne and Osbourn (2001) and Lau and Hamer (1996)
Δapf1	App differentiation	No	–	ND	–	Silué et al. (1998) (Sesma, unpublished)
Δpmk1	MAP kinase (MAPK)	No	–	No	–	Dufresne and Osbourn (2001) and Xu and Hamer (1996)
Δmps1	MAP kinase (MAPK)	No	–	No	–	Xu et al. (1998)) (Sesma, unpublished)
Leaf specific						
alb1, *buf1*	Melanin synthesis	No	–	Yes	+++	Chumley and Valent (1990), Dufresne and Osbourn (2001) and Sesma and Osbourn (2004)
ΔcpkA	cAMP signalling	No	–	Yes	++	Sesma and Osbourn (2004) and Xu et al. (1997)
Δigd1	Invasive growth	Yes	+	Yes	+	Balhadere et al. (1999) and Dufresne and Osbourn (2001)

(continued)

Table 4.3 (continued)

M. oryzae strains	Targeted gene function	APP[a]	Leaf symptom[b]	HY[c]	Root symptom	References
Δmet1	Methionine biosynthesis	Yes	+	Yes	+	Balhadere et al. (1999) and Dufresne and Osbourn (2001)
Δgde1	Glycerophosphodiesterase	Yes	++	Yes	+++	Balhadere et al. (1999) and Dufresne and Osbourn (2001)
Δmpg1	Hydrophobin	rd	+	Yes	+++	Talbot et al. (1993) (Sesma, unpublished)
Δnpr1	Nitrogen metabolism	Yes	–	ND	+++	Dufresne and Osbourn (2001) and Lau and Hamer (1996)
Δpth11	Seven transmembrane receptor	rd	+	Yes	+++	DeZwaan et al. (1999) (Sesma, unpublished)

[a]APP, appressoria
[b]scoring system: –, no symptoms; +, strong reduction; ++, weak reduction; +++, wild-type symptoms
[c]*HY* hyphopodia, *N*D not determined, *rd* reduced

4.8.1 Rice Blast Underground Infection and Arbuscular Mycorrhizal Symbiosis

There are similarities between *M. oryzae* and the ancient mycorrhizal associations. A global transcriptome profile carried out with the arbuscular mycorrhizal fungus *Glomus intraradices* and two different root-infecting fungal pathogens (*M. oryzae* and *Fusarium moniliforme*) during root infection has demonstrated the presence of common rice genes equally expressed in all three associations. This indicates a common response of rice to fungal invasion (Guimil et al. 2005). A larger set of different genes are shared between the symbiont *G. intraradices* and *M. oryzae* than between the *G. intraradices* and the necrotroph *F. moniliforme*, as expected for the biotrophic nature of *M. oryzae*. From the fungal perspective, there are also common protein domains shared by both *M. oryzae* and the symbiont *G. intraradices* implicated in root colonisation, suggesting a conservation and expansion of protein families with root colonisation-related functions (Heupel et al. 2010). This is the case for the ERA-like GTPase Erl1 of *M. oryzae* and the Gin1 protein from the symbiont *G. intraradices*. The root disease symptoms defects of *M. oryzae Δerl1* mutant are restored by reintroduction of the *G. intraradices GIN1* gene in *Δerl1*. Interestingly, the expression of the *G. intraradices* symbiotic-related gene *SP7* into

M. oryzae can decrease its necrotic behaviour on rice roots, indicating the general ability of *G. intraradices* Sp7 protein to contribute to the development of the biotrophic status of *G. intraradices* and *M. oryzae* (Kloppholz et al. 2011).

Mitochondrial respiratory activity of symbiotic fungi is stimulated by root exudates (Tamasloukht et al. 2003). Similarly, evidences suggest that mitochondrial respiration is also important for root colonisation of fungal pathogens. The *MgFOW1* gene plays an important role during *M. oryzae* invasion of root cortical cells, but it is dispensable for leaf infection (Sesma and Osbourn 2004). Fow1 was initially identified in the fungal pathogen *Fusarium oxysporum* as a protein required for colonisation of vascular tissues (Inoue et al. 2002). Fow1 is a mitochondrial carrier protein that shares close sequence similarity with the yeast protein YHM2p required for tricarboxylic acid transport. *M. oryzae Δmgfow1* mutants, like *Δfow1* mutants in *Fusarium oxysporum*, are unimpaired in their ability to utilise glycerol as a carbon source in contrast to yeast *Δyhm2* "petite" mutants, and deletion of *MgFOW1* gene has no effect in fungal growth on a range of rich and minimal media and conidiation, indicating that MgFow1 is dispensable for saprophytic growth. YHM2p associates with mtDNA in vivo and is implicated in replication and segregation of yeast mitochondrial genomes. Maintenance of mtDNA during cell division is essential for progeny to be respiratory competent. In addition, mitochondrial status is sensed by eukaryotic cells through retrograde signalling, a pathway of communication from mitochondria to the nucleus under normal and pathophysiological conditions that regulate changes in nuclear gene expression (Galluzzi et al. 2012). These changes lead to a reconfiguration of metabolism to adapt cells to defects in mitochondria. The function of Fow1-like proteins in phytopathogenic fungi is not known. MgFow1 has the potential to act as a bifunctional protein (mitochondrial carrier and mtDNA-binding protein). Elucidation of Mgfow1 function will represent an important step towards understanding invasion mechanisms of roots and vascular tissues in *M. oryzae*. The relationship between senescence and mitochondrial respiratory activity is found in ascomycetes (*P. anserina*, *N. crassa*), and further investigation in this area may help to clarify the function of the MgFow1 protein during *M. oryzae* underground infection.

4.9 Concluding Remarks

In the past years, exquisite molecular and cellular approaches have been developed to understand critical processes underlying *M. oryzae* pathogenicity. However, *M. oryzae* shows a rapid evolution of host specificity by diverse mutational events, and achieving durable blast resistance represents a challenge. Climate change is likely to alter the geographical range of fungal pathogens, and cereal infection may become more widespread and unpredictable. A clear example of this is the emerging blast disease on wheat in South America (Cruz et al. 2012). As a result of climate change, Europe may become a viable environment for *M. oryzae* on wheat, our staple cereal crop. Preventing methods and improving protection of

staple cereal crops will become vital during the following years. Undoubtedly, a better understanding of *M. oryzae* plant colonisation will have positive implications for the food security and economic stability of rice- and wheat-dependent populations worldwide. It will also have important implications for the development of new strategies for plant breeding and durable disease control. Fungal root infection processes are poorly understood within the Magnaporthaceae family due to the genetic intractability of root-infecting strains of *G. graminis* (take-all fungus) and *M. poae* species. Certainly, the dissection of *M. oryzae* root infection process will contribute to understand root infection mechanisms undertaken by fungal species of the Magnaporthaceae family.

4.10 Fungal Databases

- *Magnaporthe grisea* genome database (Broad Institute): http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html
- *Magnaporthe* comparative database (Broad Institute): http://www.broadinstitute.org/annotation/genome/magnaporthe_comparative/MultiHome.html
- Ensembl fungi: http://www.fungi.ensembl.org/Magnaporthe_oryzae/Info/Index
- FungiDB, an integrated functional genomics database for fungi: http://www.fungidb.org/fungidb/
- *M. oryzae* EST database (NIAS): http://www.mg.dna.affrc.go.jp/
- COGEME EST Database: http://www.ri.imb.nrc.ca/cogeme/index.html
- *M. grisea* MPSS database (Massively Parallel Signature Sequencing): http://www.mpss.udel.edu/mg/
- Orygenes DB: an interactive tool for rice reverse genetics http://www.orygenesdb.cirad.fr/
- Oryzabase: Integrated Rice Science Database http://www.shigen.nig.ac.jp/rice/oryzabase/top/top.jsp
- MGOS, *Magnaporthe grisea—Oryza sativa* interaction database: http://www.mgosdb.org/
- PHI base (Pathogen–Host Interaction database) offers molecular and biological information on genes involved in host–pathogen interactions. http://www.phi-base.org/
- Fungal Secretome Database: http://www.fsd.riceblast.snu.ac.kr/index.php?a=view
- Comparative fungal genomics platform: http://www.cfgp.riceblast.snu.ac.kr/main.php
- *M. oryzae* T-DNA analysis platform: http://www.atmt.snu.ac.kr/ and http://www.tdna.snu.ac.kr/
- Fungal transcription factor database: http://www.ftfd.snu.ac.kr/index.php?a=view
- Fungal Nomenclature databases: http://www.indexfungorum.org/ and http://www.mycobank.org/

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Chapter 5
Aspergillus: Genomics of a Cosmopolitan Fungus

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5.1 *Aspergillus* Taxonomy and Lifestyles

The genus *Aspergillus* consists of more than 250 species including both pathogenic and beneficial species (Geiser et al. 2008). Several species are opportunistic pathogens of plants, animals, and humans (e.g., *A. fumigatus*, *A. terreus*) and/or produce toxins, such as aflatoxins and ochratoxins (e.g., *A. flavus*, *A. parasiticus*). On the other hand, several species are widely used in industrial applications for the production of foods, organic acids, and a large variety of enzymes (e.g., *A. niger*, *A. aculeatus*, *A. oryzae*). The broad relevance and economic importance of the genus have pushed it to the forefront of fungal research, with one of the largest academic and industrial research communities dedicated to this genus.

Members of the genus *Aspergillus* are characterized by the unifying feature of the "aspergillum," an asexual reproductive structure, and form a broadly monophyletic group. However, there is surprisingly large taxonomic divergence in the group both in terms of morphology (e.g., contrasting sexual states) (Geiser et al. 2008) and

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_5,
© Springer-Verlag Berlin Heidelberg 2013

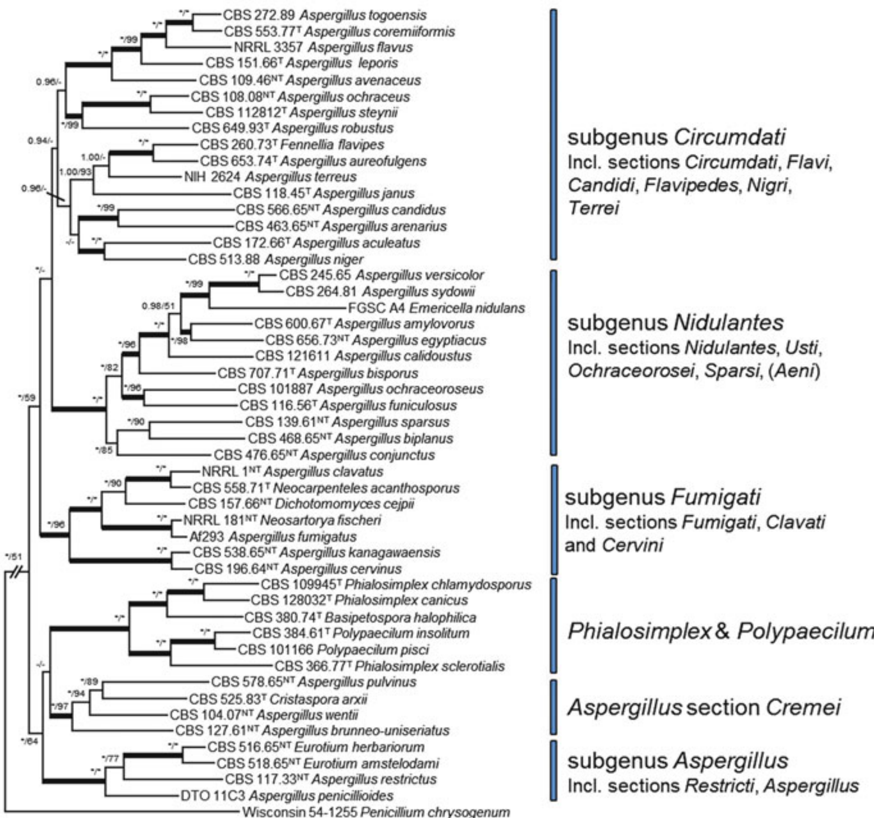

Fig. 5.1 Best-scoring maximum likelihood tree presenting current *Aspergillus* phylogeny. Based on Houbraken and Samson (2011)

phylogenetic difference (e.g., up to 30 % or greater genome sequence divergence) (Galagan et al. 2005). The first record of *Aspergillus* can be found in Micheli's Nova plantarum genera (Micheli 1729), but a more detailed description of the Aspergilli did not appear until the middle of the nineteenth century. The Aspergilli belong to the family of *Trichocomaceae* of the order *Eurotiales* of the class *Eurotiomycetes* of the phylum Ascomycota, and *Aspergillus* is a sister genus to *Penicillium* and *Talaromyces*. A recent study provided a detailed overview of the taxonomic relations between these species (Houbraken and Samson 2011). Based on this, the genus *Aspergillus* contains the subgenera *Circumdati* (including sections *Circumdati*, *Flavi*, *Candidi*, *Flavipedes*, *Nigri*, and *Terrei*), *Nidulantes* (including sections *Nidulantes*, *Usti*, *Ochraceorosei*, and *Sparsi*), *Fumigati* (including section *Fumigati*, *Clavati*, and *Cervini*), and *Aspergillus* (including sections *Restricti* and *Aspergillus*), as well as section *Cremei* and fungi originally named *Phialosimplex* and *Polypaecilum* (Fig. 5.1).

Several sections of this genus have received wide attention in research and society due to their medical, agricultural, or biotechnological importance. The section *Nigri*, also known as the black Aspergilli, is well known for its relevance to food mycology, medical mycology, and biotechnology. This section contains major spoilage fungi of various food products but also species that are used industrially for the production of enzymes and metabolites (Varga et al. 2000). In contrast, *A. fumigatus* is generally considered to be the most harmful opportunistic filamentous fungus to people (Pringle et al. 2005). It is not only a major opportunistic pathogen but also causes allergies globally (Denning 1998; Latge 1999). The best known species from the section *Terrei* is *A. terreus*, which is used for the production of itaconic acid and itatartaric acid (Bigelis and Arora 2009). It also produces a number of valuable secondary metabolites, such as the cholesterol-lowering drug lovastatin (Alberts et al. 1980) and the antitumor metabolites asterriquinone and terrein (Kaji et al. 1998; Arakawa et al. 2008).

The availability of genome sequences for several Aspergilli has enabled a genome-wide look at differences between closely and more distantly related species. An initial study on six Aspergilli demonstrated that genome comparisons follow the current view on *Aspergillus* taxonomy, in that closely related species (e.g., *A. oryzae* and *A. flavus* or *A. fumigatus* and *N. fischeri*) are also highly similar in genome content and organization (Rokas et al. 2007). Such analyses have also been done for larger sets of fungi (Wang et al. 2009), in which the composition vector method was used to create a fungal phylogeny. The relative position of the *Aspergillus* species included in this tree matches well with the more extensive phylogeny of the *Aspergillus* genus and its sister genera (Houbraken and Samson 2011). The availability of genomes also offers the possibility of quickly developing novel strain typing methods to discriminate between multiple isolates of a species or to aid in the recognition and identification of a species (Klaassen and Osherov 2007).

Many Aspergilli have a global distribution, although reports on this are strongly affected by the number of isolates that were described for any given species, which in itself is affected by the medical, agricultural, or biotechnological relevance of the species. Based on the isolates present in the CBS collection (http://www.cbs.knaw.nl/), global distribution can be observed for most of the well-studied Aspergilli, such as *A. nidulans*, *A. flavus*, *A. fumigatus*, *A. niger,* and *A. terreus*. Evaluating the biotope of the *Aspergillus* isolates in the CBS collection demonstrates that the majority was isolated from soil, followed by food and feed spoilage and indoor environments. Only a small number of isolates was obtained from wood, leaves or litter, or dung. A significant number of medical isolates is also present, but the majority of these are from *A. fumigatus*, confirming its status as the main human pathogen of this genus.

Despite not being considered major plant pathogens, several Aspergilli do affect plants and can cause significant spoilage problems on plant products, such as rotting, development of off-odors and off-flavors, and discoloration (Perrone et al. 2007). The most significant food problem originating from Aspergilli is the production of mycotoxins, such as aflatoxin and ochratoxin A (Varga et al. 2004).

The most important aflatoxin-producing species belong to the section *Flavi*, such as *A. flavus* and *A. parasiticus* (Bennett and Klich 2003), while ochratoxin is mainly produced by sections *Circumdati* and *Nigri* (Frisvad et al. 2004; Samson et al. 2004)

Some species, such as *A. flavus*, can also infect a broad range of agricultural crops, including monocots and dicots (St Leger et al. 2000). *A. flavus* is also the second most common (after *A. fumigatus*) fungus causing aspergillosis in immune-compromised humans (Denning et al. 1991). This negative reputation is a strong contrast with the positive reputation of its close relative *A. oryzae* that is widely used in the Japanese fermentation industry and as an industrial enzyme producer (Kobayashi et al. 2007). A recent study addressed the potential for biotechnology of *A. flavus* and demonstrated that it has a large selection of genes related to the production of secondary metabolites and carbohydrate-degrading enzymes (Cleveland et al. 2009). More recently, it was suggested that *A. oryzae* has evolved from *A. flavus* by domestication and selection by humans (Gibbons et al. 2012).

In soil, Aspergilli mainly use plant biomass as a carbon source and produce extensive enzyme systems to degrade this substrate (de Vries and Visser 2001). These enzymes have also found applications in a wide range of applications (de Vries and Visser 2001; de Vries 2003; van den Brink and de Vries 2011), and are discussed in more detail in Sect. 5.5.3.

5.2 Development and Current Status of *Aspergillus* Genomics

Aspergillus genomics initiated with the genome sequence of *A. nidulans* that was initially performed by Monsanto and later finished by the Broad Institute. During this period, also the genome sequences of *A. oryzae* and *A. fumigatus* were obtained, and publication of these first three genomes occurred in the same issue of Nature (Galagan et al. 2005; Machida et al. 2005; Nierman et al. 2005b). Within a relative short time after that, genome sequences also became available for *A. niger* (Pel et al. 2007; Andersen et al. 2011), *A. clavatus* (Fedorova et al. 2008), *A. terreus*, *A. flavus* (Payne et al. 2006), and *N. fischeri* (Fedorova et al. 2008), as well as a second genome for *A. fumigatus* (Nierman et al. 2005b; Fedorova et al. 2008). More recently, *A. carbonarius*, *A. aculeatus,* and *Eurotium herbariorum* (*Aspergillus herbariorum*) have been sequenced by the Joint Genome Institute (JGI) of the Department of Energy of the USA, and draft genomes have been published for *A. kawachii* (Futagami et al. 2011) and *A. sojae* (Sato et al. 2011). A project of the Community Sequencing Program (CSP2011) (Grigoriev et al. 2011) of the JGI involved sequencing of an additional eight Aspergilli (*A. brasiliensis*, *A. tubingensis*, *A. acidus*, *A. versicolor*, *A. sydowii*, *A. wentii*, *A. glaucus*, *A. zonatus*), most of which are already available through the JGI MycoCosm website (http://genome.jgi.doe.gov/programs/fungi/index.jsf) (Grigoriev et al. 2012). A recently granted CSP2013 project will sequence an additional three Aspergilli (*A. ochraceus*, *A. sparsus*, *A. cervinus*) as well as

12 Penicillia (*P. glabrum*, *P. citrinum*, *P. brasilianum*, *P. simplicissimum*, *P. araracuaraense*, *P. rapidoviride*, *P. oxalicum*, *P. corylophilum*, *P. anczewskii*, *P. jamesonlandense*, *P. brevicompactum*, *P. funiculosum*), *Talaromyces thermophilus,* and *Rasamsonia emersonii*. Together with the already available genomes of the sister genera from *Aspergillus*, *Penicillium rubens* (van den Berg et al. 2008), *Penicillium chrysogenum* (sequenced by JGI), *Talaromyces stipitatus,* and *Talaromyces marneffei* (Woo et al. 2011), this provides the most detailed set of genomes for any group of filamentous fungi (Gibbons and Rokas 2013).

There is relative high variability in the genomes of different *Aspergillus* species, which correlates with the taxonomic distance of the species (Rokas et al. 2007). Interestingly, the difference between the genomes of *A. oryzae* and *A. flavus* is similar to the difference between the two published *A. niger* genomes (Rokas et al. 2007). Comparison of the two *A. niger* genomes revealed several genome rearrangements, deletions, horizontal gene transfer, and a high frequency of single nucleotide polymorphisms (SNPs), demonstrating the high level of genome evolution in these strains (Andersen et al. 2011). High variation in *Aspergillus* genomes was also detected using synteny analysis of *A. oryzae*, *A. nidulans,* and *A. fumigatus*, which demonstrated that the *A. oryzae* genome has a mosaic structure consisting of syntenic and non-syntenic blocks, possibly caused by genome-wide gene duplications events in *A. oryzae* (Machida et al. 2008).

The availability of several *Aspergillus* genome sequences has stimulated the development of several databases and tools dedicated to (post-)genomic studies of the genus. The first example of this was the comparative Aspergillus server at the Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). This was the first online resource that enabled comparative analysis of multiple *Aspergillus* genomes using various tools. Two other resources for *Aspergillus* genomics are CADRE (http://www.cadre-genomes.org.uk) and Aspergillus Genomes (http://www.aspergillus-genomes.org.uk) and AspGD (http://www.aspgd.org). CADRE, the Central Aspergillus Data Repository, was initiated in 2001 to support the international *Aspergillus* research community by gathering all genomic information for this genome in one public resource. While it initially focused strongly on medical issues, it has grown to a more general resource that currently contains genomes and related data for eight Aspergilli (Mabey Gilsenan et al. 2012). It has tools for BLAST and browsing, gene analysis, and comparisons between genomes. Aspergillus Genomes is a union of CADRE and the *Aspergillus*/Aspergillosis website and focuses more on medical issues in relation to *Aspergillus* genomics (Mabey Gilsenan et al. 2009). AspGD was developed as comprehensive *Aspergillus* database through a collaborative effort of the University of Maryland and Stanford University and aims to provide the scientific research community with a web-based resource of structurally and functionally annotated genomes, supported by manual literature-based curation (Arnaud et al. 2012). Tools in AspGD include comparative and synteny analysis, BLAST, ORF histories, and possibilities for improvement of gene structure. Currently, AspGD hosts genomes for 12 Aspergilli, with more to be added in the coming year.

The comparative *Aspergillus* genome project that is running at JGI has also created a genome analysis infrastructure for these fungi within the MycoCosm portal of the JGI (http://genome.jgi.doe.gov/programs/fungi/index.jsf) (Grigoriev et al. 2012). Currently, MycoCosm contains genomes for 11 Aspergilli with more coming out as a result of the ongoing projects (see above).

Following the availability of *Aspergillus* genomes, post-genomic technologies such as transcriptomics and proteomics have been developed. Various types of microarrays have been generated for Aspergilli, ranging from glass slides to Affymetrix gene chips. While they have been mainly used for scientific studies, they have also found applications in monitoring fermentations (Maeda et al. 2004). More recently, RNAseq has started to replace microarrays in transcriptomics studies, due to the significant reduction in costs of RNA sequencing. Proteomics has been applied in several *Aspergillus* research areas. Two recent reviews of proteomics studies for several Aspergilli demonstrated the breadth of these studies, covering intracellular, extracellular, and cell wall proteomes and addressing topics from stress response to polysaccharide degradation and modification (Kim et al. 2008; Kniemeyer 2011). While the majority of the proteomics studies are aimed at specific scientific questions, proteomics can also aid in genome annotation and gene model validation. A recent study in *A. niger* mapped 405 identified peptide sequences to 214 genomic *loci,* and for 13 % of these *loci,* new or improved gene models could be constructed based on the proteomics data (Wright et al. 2009).

These resources have had a tremendous effect on many aspects of *Aspergillus* research, and some examples are listed below. Proteomics and transcriptomics analyses of the response of *A. fumigatus* to antifungals, such as Caspofungin (Cagas et al. 2011), Artemisinin (Gautam et al. 2011), and Coumarin (Singh et al. 2012), have identified potential drug targets and biomarkers to assess the relative efficacy of drug therapy. Such studies have also opened the door toward systems biology studies of *A. fumigatus* infection (Albrecht et al. 2011). In *A. nidulans* a detailed analysis of metabolic genes resulted in a metabolic pathway model that improved the functional annotation of the genome (David et al. 2008). A combination of genome-scale modeling with comparative genomics and transcriptomics revealed insights into the evolution and role of the highly efficient acidification by *A. niger* of its local environment (Andersen et al. 2009). The discovery of genomic evidence for sex in species with no known sexual stage has already resulted in the discovery of a sexual cycle in *A. fumigatus* (O'Gorman et al. 2009) and other species are currently under investigation. Genome mining of the Aspergilli has not only discovered many putative secondary metabolite gene clusters (Bok et al. 2006) but also demonstrated that several of the secondary metabolite gene clusters in *A. nidulans* are "silent" (not expressed under any condition tested) (Scherlach and Hertweck 2006). Activation of such a silent cluster was reported resulting in the discovery of novel metabolites (Bergmann et al. 2007). Genome comparison in combination with biological studies also highlighted differences between the Aspergilli, such as in regulation and metabolism of plant-related carbon sources for *A. niger* and *A. nidulans* (Battaglia et al. 2011;

Christensen et al. 2011; Gruben et al. 2012). A detailed genome analysis enabled an inventory of the polysaccharide-degrading potential of *A. niger* (Andersen et al. 2012), while comparative genomics highlighted the differences between *A. niger*, *A. oryzae,* and *A. nidulans* for this topic (Coutinho et al. 2009).

To illustrate in more detail the impact and potential of genomics for *Aspergillus*, three important *Aspergillus* research topics will be discussed in more detail in the sections below: plant biomass utilization, signal transduction, and secondary metabolism.

5.3 Plant Biomass Utilization

Estimating the total amount of plant biomass on earth is very difficult. Information on plant biomass is available from a mixture of sources; the data are often regional or national, based on different methodologies and not easily accessible. A database of average biomass per country is produced by the Food and Agriculture Organization of the United Nations (FAO). Plant biomass includes woody and nonwoody vegetation, crops, waste, or by-products from agriculture or from industries but also domestic garbage. Plant biomass is the most renewable material on earth and, with the depletion of fossil energy, expected to be a valuable resource for sustainable energy and chemicals. By 2020, the European Union aims to have 20 % of total energy consumption from renewable energy sources with 10 % only for transport which is a very challenging commitment. In 2005, 66.1 % of renewable energy produced in the EU was from the breakdown of biomass (http://europa.eu/legislation_summaries/energy/renewable_energy/l27065_en.htm). In 2011, biomass fuels provided only about 4 % of the energy used in the United States (http://www.eia.gov/energyexplained/index.cfm?page=biomass_home). European projects such as Hype (http://www.helsinki.fi/hype), Disco (http://www.disco-project.eu), and Nemo (http://nemo.vtt.fi) are meant to develop novel-integrated concepts for hydrolysis and fermentation of lignocellulosic material to obtain bioethanol from second-generation biomass, i.e., from plant material that does not compete with food production.

5.3.1 Plant Cell Wall Polysaccharides

Fungi secrete a broad range of enzymes, breaking down the plant cell wall polymers into monomers that can be further taken up and metabolized as carbon sources. The main components of the plant cell wall are polysaccharides, cellulose, hemicellulose, and pectin, linked to each other and closely associated with lignin, a semi-random, three-dimensional aromatic polymer. The lignin fraction is very recalcitrant to degradation due to its complex structure and high molecular mass. The presence of lignin limits the saccharification yields and is responsible for the darkness of pulp

in high-quality white paper production. A limited number of enzymes are known to depolymerize lignin (Chen et al. 2012). These are oxidative enzymes such as peroxidases and laccases, and although some are present in *Aspergillus* genomes, delignification is not a well-known ability of the Aspergilli (Pel et al. 2007). Therefore, the focus is on the utilization and valorization of plant polysaccharides. Cellulose is the main carbohydrate produced by plants and consists of a linear polysaccharide of β-1.4-linked D-glucose units organized in microfibrils (Lavoine et al. 2012). Hemicellulose is considered to be the second most abundant plant cell wall component and consists of heterogeneous polysaccharides such as xylan, xyloglucan, galacto-, and galactoglucomannan of which the amount varies depending on plant origin (Peng et al. 2012). Pectin also consists of diverse and complex polysaccharides, such as homogalacturonan, rhamnogalacturonan-I, and substituted galacturonans (Hilz 2007).

Each of these polysaccharides has a broad range of applications. The worldwide production of cellulose as a biopolymer, for instance, is estimated to be between 10^{10} and 10^{11} tons each year. Cellulose, hemicelluloses, and pectins can be hydrolyzed into hexoses and pentoses and further fermented to produce bioethanol. Monosaccharides or partially hydrolyzed polysaccharides can be converted into added-value chemicals such as xylitol and furfural (Peng et al. 2012). Furfural is used as a building block for chemical synthesis (Pace et al. 2012), and vanillin is used in a broad range of flavors for foods, confectionery, and beverages (approximately 60 %); as a fragrance ingredient in perfumes and cosmetics (approximately 33 %); and for pharmaceuticals (approximately 7 %) (Priefert et al. 2001). Besides being more environmentally friendly, enzymatic treatments of the polysaccharides target specific linkages while chemical or mechanical treatments are less specific (Benoit et al. 2006). Carbohydrate-active enzymes acting on the plant polysaccharides are organized in several families based on amino acid sequence of the structurally related catalytic modules (http://www.cazy.org) (Henrissat 1991; Cantarel et al. 2009). Three different fungal hydrolases (β-1,4-endoglucanase, cellobiohydrolase, β-1,4-glucosidase) divided over seven families (GH1, 3, 5, 6, 7, 12, 45) are acting on cellulose, while 15 types of fungal enzymes, including hydrolases, lyases, and esterases divided over 21 families, are acting on pectin (van den Brink and de Vries 2011). Based on the annotated genomes and the CAZy database, transcriptomics and proteomics have become interesting tools to study the different sets of enzymes involved in polysaccharide degradation.

5.3.2 Aspergilli: Industrial and Model Organisms

Aspergilli are used for industrial production of enzymes and metabolites. The workhorse *A. niger,* for instance, produces high amounts of citric acid and is well known for its efficient secretion of glucoamylase (up to 20 g/L) (Finkelstein 1987). *A. oryzae* plays a central role in Asian traditional fermented condiments like soy sauce, miso, or sake. In addition to being industrial organisms, these two Aspergilli

became model organisms, together with *A. nidulans*, and have been for more than 50 years (Rittenour et al. 2009). These Aspergilli are relatively easy to grow and maintain in a restricted environment. Nutrients and growth conditions are well known. Their growth rate is relatively fast within the fungal kingdom, and they are recognized by the United States Department of Agriculture as a Generally Regarded As Safe (GRAS) organisms which makes them interesting to study their development and be a reference for other fungi. Moreover, many molecular tools were developed to transform them. Therefore, they are model organisms to study eukaryotic protein secretion (Baker 2006). The *fungal growth* database (http://www.Fung-Growth.org) displays the growth profiles of 42 Aspergilli grown on a broad range of substrates from monosaccharides to more complex substrates such as crude plant biomass. These substrates induce different responses from the Aspergilli. Therefore, the analysis of the transcripts and/or the secretome provides insights on how these fungi use their enzymes to efficiently degrade the substrate. Recently, the extracellular proteome of *A. nidulans* grown on sorghum stover was studied at different time points (Saykhedkar et al. 2012). A total of 294 extracellular proteins were identified including cellulases, hemicellulases, pectinases, chitinases, and lipases. This 14-day time course study revealed that most of the enzymes are already secreted at day 1, and only the relative abundance of the enzymes changes over the time. These data suggest that the breakdown of the plant polysaccharides is simultaneously done. The presence of main chain acting enzymes, accessory enzymes, and protein that could contribute to an efficient hydrolytic system make *A. nidulans* capable of degrading the major polysaccharides in sorghum without chemical pretreatment (Saykhedkar et al. 2012).

5.3.3 Comparative Proteomics: In Silico Versus In Vivo

Based on the presence of signal peptides in almost all secreted proteins, computational approaches coupled to mass spectrometric analysis have been used to predict the secretome of *A. niger*, *A. oryzae,* and *A. nidulans* (Tsang et al. 2009; Braaksma et al. 2010). The proteome size varies between strains and species and does not necessarily correlate with the genome size. *A. oryzae* has the largest genome but does not have the highest number of protein-coding genes nor signal peptide predictions (Table 5.1). The secretome of *A. niger* grown under six conditions, glucose, sorbitol, glycerol, birchwood xylan, citrus pectin and locust bean gum, during 2 days, identified a total of 222 proteins. Fewer than 15 % of these have been biochemically characterized (Tsang et al. 2009). Most of the putative enzymes involved in the hydrolysis of cellulose, xylan, and arabinan were secreted in all six conditions while mannanases and pectinases appeared to be more substrate dependent. Mannanases were only detected on locust bean gum and pectin, and pectinases were mainly detected on pectin. When the secretome of *A. niger* grown on galacturonic acid was compared to the secretome of the same strain grown on

Table 5.1 Genome size, proteome size, and signal peptide predictions of four *Aspergilli*

Species	*A. niger* CBS 513.88	*A. niger* ATCC 1015	*A. oryzae* RIB40	*A. nidulans* FGSC A4
Genome size (Mb)	33.93[a]	34.85[a]	37.6[b]	30.06[c]
Protein CDS	14,086[d]	11,197[e]	10,406[e]	10,665[e]
SignalP3 NN	1,831	1,540	1,751	1,469

Adapted from Braaksma et al. (2010)
[a]Andersen et al. (2011)
[b]Machida et al. (2005)
[c]Galagan et al. (2005)
[d]Number obtained from the Refseq section of GenBank
[e]Number obtained from http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html

sorbitol, the pectinolytic genes were clearly induced. For instance, pectin lyase A; exopolygalacturonase A, B, C, and X; as well as putative arabinofuranosidase and pectin acetylesterase were detected on galacturonic acid and not on sorbitol (Braaksma et al. 2010).

5.3.4 *Limitations in Protein Identification*

It has been shown that some secreted proteins are retained in the cell wall after being secreted (Levin et al. 2007). Some proteins secreted in solid-state cultures were found to be trapped in the cell wall during submerged culture, such as most of the secreted α-amylase and β-glucosidase of *A. oryzae* when grown in wheat bran submerged cultures (Oda et al. 2006). A recent study shows the positive effect of the cycloheximide on protein release into the medium. *A. niger* was grown on D-xylose, and the secreted proteins were analyzed within the different zones of the mycelium, from center to the periphery of the colony grown on solid medium. 187 proteins were identified from cultures without cycloheximide, of which 98 passed the filtering criteria, while 216 proteins were identified in the presence of cycloheximide, of which 148 passed the filtering criteria (Krijgsheld et al. 2012).

Incomplete peptide sequence databases, due to missed protein-encoding genes or gene model errors, affect the number of proteins identified in proteomics, but the method used also has an influence. The precipitated extracellular proteins from the culture filtrate can be directly digested by trypsin, followed by MALDI-TOF/MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry), or first be separated by 2-D or 1-D SDS gel electrophoresis, which is the most common technique for protein extraction. Extra steps such as an in-gel deglycosylation with PNGase F may increase the number of proteins that can be identified by MS (Bouws et al. 2008). However, a large number of secreted proteins are lost or remain unidentified. The hydrolysis of alkali pretreated rice straw by *A. fumigatus* has been studied using zymograms, followed by proteomics. The cocktail of enzymes

produced by a thermotolerant *A. fumigatus* strain resulted in >90 % saccharification of the substrate. β-glucosidase, cellobiohydrolase, xylanase, endoglucanase, and acetyl esterase activities were identified by zymograms. Secretome characterization revealed a combination of a variety of glycosyl hydrolases (Sharma et al. 2011). Although the proteome of *A. fumigatus* has been previously documented in a medically related context (Carberry and Doyle 2007), this study is the first report on *A. fumigatus*'s CAZymes.

5.3.5 Intracellular Proteome and Transcriptomics

Other approaches such as microsomal proteome analysis of *A. niger* gave more information on intracellular membrane traffic and protein secretion upon cellulase and hemicellulase induction by D-xylose or amylase induction by D-maltose compared to sorbitol (de Oliveira and de Graaff 2010; de Oliveira et al. 2010). Exploration of the intracellular proteome of *A. oryzae* and *A. niger* resulted in the identification of a much higher number of proteins. 522 proteins were identified from *A. oryzae*, amongst which 451 proteins are potentially involved in the production of soy sauce flavor (Zhao et al. 2012). From *A. niger* grown on D-xylose and maltose, differences found in the two intracellular proteomes were related to the first steps in carbon catabolism. The utilization of D-xylose or maltose affected the composition of the secretome but had a minor influence on the composition of the intracellular proteome. On the other hand, the different conditions of the cultures, in particular pH and the aeration factor, had a strong effect on the intracellular proteome (Lu et al. 2010).

Using transcriptomics, an overview of the expression of genes encoding (extracellular) plant cell wall-degrading enzymes as well as the genes involved in the related (intracellular) metabolic pathways is obtained. Transcriptome analysis of *A. niger* grown on sugarcane bagasse, a bioethanol substrate, revealed the genes specifically induced by this substrate. In addition to the predicted cellulases and hemicellulases, many genes encoding unknown functions had increased expression. Transporter-encoding genes potentially involved in oligosaccharide transport were also expressed (de Souza et al. 2011). Hitherto the limiting step in a total and efficient plant biomass valorization is the right enzyme cocktail. Combining the great ability of the Aspergilli to grow on a wide range of substrates with the post-genomic tools will help in designing novel-tailored enzymatic cocktails for biotechnology as well as understand better the interactions between the fungi and their environment (de Vries et al. 2011).

5.4 Signal Transduction

The complete genome sequence of several *Aspergillus* species published over the last 7 years has provided a valuable tool for the identification and deeper characterization of the gene function in this important genus. By these means, it has also led to an unprecedented understanding of the molecular basis of some of the most striking features of the fungal organisms and *Aspergilli* itself, i.e., their ability to sense the environment and to colonize diverse ecological niches (in the case of the saprobic lifestyle species) or to recognize and to adapt to the host's milieu (in the case of the pathogenic species). These events ultimately facilitate survival under different temperature and nutrient limitation and to overcome the host intrinsic defenses. Independently of the environments, the ability of the fungal cells to interact with them highly relies on their capacity to recognize the external boundaries of the cells and properly respond to the signals that emanate from this environment. All this information must be gathered together by the fungal cell by means of the well-orchestrated multiple circuits of a signal transduction system. Such systems enable the effector proteins to launch the appropriate response for cell homeostasis, proliferation, and quiescence, which finally represents an overall cell metabolic adaptation. Fungi are organisms for which adaptation is mandatory, and it is this capability that probably makes these organisms so versatile. Understanding how these diverse signaling networks are organized in the fungal cell allowing such versatility is an interesting point that *Aspergilli* genome exploitation can help to unravel.

Many of the mechanisms that connect signaling proteins into networks are thought to be highly modular; i.e., the catalytic core of a signaling pathway is physically and functionally separable from molecular domains or motifs that determine its linkage to both inputs and outputs. This high degree of modularity may make these systems more evolvable, creating the possibility for new pathways with different cell behaviors upon competitive or hostile environments [for a review see Bhattacharyya et al. (2006)]. However, the signaling pathways and output responses to external signals show a high degree of conservation and different signaling factors contribute to fungal adaptation. Environmental signals such as temperature, carbon source, pH, oxygen content, and mechanical or physical elements or soluble factors such as drugs or xenobiotics are elements that can trigger a variety of signaling pathways. Filamentous fungi are more tolerant to various conditions than yeasts and are thus expected to have more extensive sensing and signaling networks. Analysis of the genome of the *Aspergillus* species demonstrated that the organization of the sensing and signal transduction systems and particularly the upstream signaling mechanisms are more complex than those found in yeasts (Abe et al. 2010). The main common pathways which are shared in all *Aspergillus* species will be highlighted in the following sections of this chapter.

5.4.1 cAMP/PKA-Signaling Cascade and G-Protein-Coupled Receptors in Aspergillus

Although the fully sequenced genomes of several *Aspergillus* species have been analyzed, little is known about the signal transduction pathways involving the heterotrimeric guanine nucleotide-binding proteins (G-proteins). In eukaryotic cells, these proteins interact with the cell surface plasma membrane proteins which are members of a large superfamily of receptors having seven membrane-spanning regions. Collectively this superfamily is named G-protein-coupled receptors (GPCR). This signal transduction cascade becomes functional after the extracellular stimuli arrive at these membrane receptors. In fungi, these stimuli reflect those related to fungal cell survival and fitness, virulence, secondary metabolite production, morphogenetic events, and so on (Bolker 1998; Lengeler et al. 2000). Heterotrimeric G-proteins are composed of three subunits α, β, and γ forming a complex (Neves et al. 2002). The functioning dynamics of the GPCR complex includes the activation of the G-protein upon ligand binding at the cell surface. Figure 5.2 represents this sensitization step generally as the various extracellular signals provide inputs from the extracellular compartment. GPCR sensitization promotes the interaction between GPCR and the GDP-Gα inactive form of the Gα-Gβ-Gγ heterotrimeric complex (black circle in Fig 5.2) resulting in Gα-GDP to Gα-GTP exchange. This alteration leads to the dissociation of Gα-GTP from the Gβ-Gγ heterodimer. Once dissociated, the Gα-GTP subunit, Gβ-Gγ, or both can amplify and propagate signals by modulating activities of a number of effector proteins causing a given cell behavior [for a review, see Neves et al. (2002)].

Through genome analysis of *A. nidulans*, *A. fumigatus,* and *A. oryzae,* it was possible to identify at least 16 putative GPCR which were grouped in nine different classes based on detailed phylogenetic studies [Lafon et al. 2006 and revised in Yu (2006) and Yu and d'Enfert (2008)]. Figure 5.2 summarizes the GPCRs, the G-protein subunits present in the genome of *Aspergillus,* and the main downstream effectors which will be discussed in this chapter. Of these 16 GPCRs, up to now, very few have been well characterized, and most studies address GprA, GprB, and GprD (Han et al. 2004; Seo et al. 2004). Deletion of *gprD* resulted in hyphal and germination defects and increased production of cleistothecia (fruiting bodies), indicating that this GPCR is probably a negative regulator of sexual development in *A. nidulans* (Han et al. 2004). In contrast, the deletion strains Δ*gprA* and Δ*gprB* resulted in reduced cleistothecia which were significantly smaller than those in a wild-type *A. nidulans* strain (Seo et al. 2004). Moreover, the double mutant Δ*gprA*/Δ*gprB* exhibited complete absence of cleistothecia (Seo et al. 2004).

The most comprehensive characterization of the G-protein subunits is described in *A. nidulans* and *A. fumigatus*. Among them, Gα of *A. nidulans* FadA presents the so-called fluffy autolytic dominant phenotype since strains carrying dominant-activating mutations of this gene have a striking phenotype of increased accumulation of aerial hyphae and lack of development and conidiogenesis which leads to

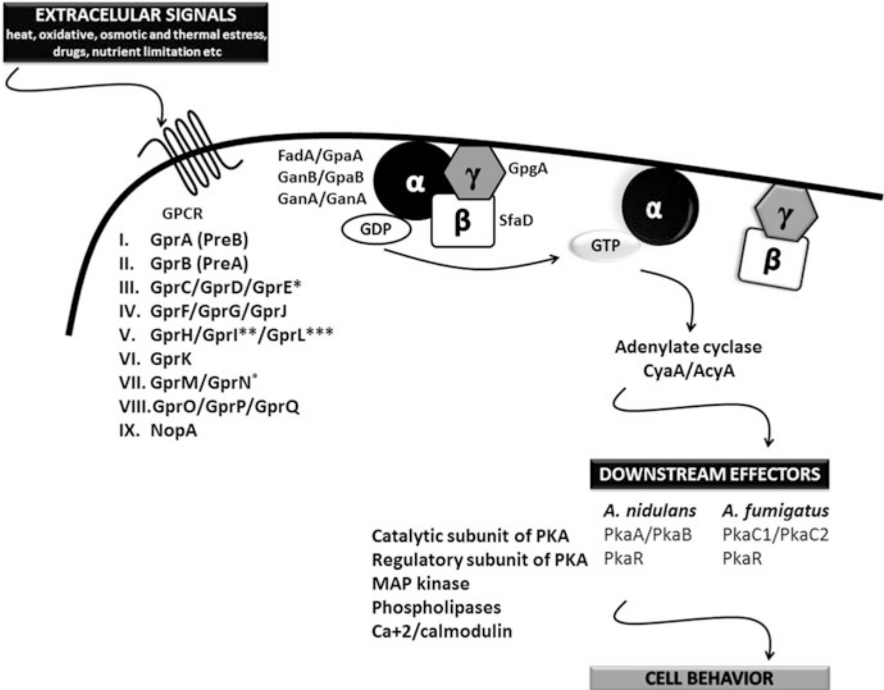

Fig. 5.2 Schematic representation of the cAMP-Protein kinase A-dependent pathway in *Aspergilli*. The figure shows the 11 families (I–XI) of GPCR (G-protein-coupled receptor) identified "in silico" for the *A. nidulans*, *A. fumigatus,* and *A. oryzae*. The description of those with functional analysis is available is in the text. The information in this figure is adapted from Lafon et al. (2006), Yu (2006), and Yu and d'Enfert (2008). *Only present in *A. nidulans*. **Not present in *A. oryzae*. ***Only present in *A. fumigatus*. The names for the three genes encoding different Gα subunits (*black circle*) are separated by a slash to indicate names in *A. nidulans* and *A. fumigatus*, respectively, in each line. The Gβ (*white rectangle*) and Gγ (*gray hexagon*) subunits encode a single gene in each organism. The genome of *A. oryzae* also contains a fourth Gα subunit named GaoC, in addition to those mentioned here [for details refer to Lafon et al. (2006)]. The protein kinase A (PKA) is activated upon the action of the second messenger cAMP (cyclic AMP) which rises in concentration inside the cell by the increased activity of the adenylate cyclase gene (*cyaA* and *acyA* in *A. nidulans* and *A. fumigatus*, respectively). Both *A. nidulans* and *A. fumigatus* have two PKA catalytic subunit-encoding genes and a single gene encoding the regulatory subunit (*pkaR*). The relevance of these genes in the cAMP-Protein kinase A-dependent pathway is discussed in the text. MAP kinase can be a downstream effector of a GPCR response, and the main MAP kinase pathways in *Aspergilli* are discussed in the Sect. 5.4.2. Phospholipases and calcium/calcineurin can also function as downstream effectors but will not be discussed in this text. The cellular behavior indicates the final element in the cell adaptation to the extracellular input signals. In general regards to an enhanced transcriptional activation of key genes which products ultimately drive the cell to adaptation and survival

complete hyphal disintegration in cultures (Yu et al. 1996, 1999; Hicks et al. 1997; Wieser et al. 1997). This phenotype indicates a clear role of *fadA* in vegetative growth and inhibition of sexual and asexual development. In contrast, an *A. fumigatus* strain containing a dominant-activating form of the *fadA* homolog, *gpaA*, also shows increased hyphal proliferation but not the fluffy autolytic phenotype (Yu et al. 2006). The Δ*gpaB* mutant from *A. fumigatus* resulted in reduced conidia production with normal growth rate but was almost avirulent in a mouse infection model (Liebmann et al. 2003, 2004).

The only pathway where a link between a G-protein and its downstream effector has been formally established is the GanB/CyaA cAMP-PKA cascade during germination (Yu and d'Enfert 2008). Hence, one of the well-studied cellular downstream effectors of the GPCR is the cAMP-PKA circuit. Signal transduction coming from GPCR activity stimulates the plasma membrane-bound enzyme adenylyl cyclase, encoded by the genes *cyaA* and *acyA* in *A. nidulans* and *A. fumigatus*, respectively, but still uncharacterized in other *Aspergilli*, such as *A. oryzae* and *A. niger*. The activity of CyaA/AcyA results in the production of the second messenger cyclic AMP (adenosine 3′,5′-cyclic monophosphate) from ATP. The increased intracellular levels of cAMP activate protein kinase A (PKA). Activation of PKA takes place by the cAMP-induced conformational change in the PKA regulatory subunits which is ultimately released from the PKA catalytic subunit (see Fig. 5.2 for gene names), allowing it to phosphorylate downstream targets at serine or threonine residues, culminating in the cellular response upon stress or other signals. In this process, which is well conserved across eukaryotes, two cAMP molecules bind to the homodimeric structure of PKA regulatory subunit (encoded by *pkaR* in Aspergilli), releasing the catalytic PKA structure (Taylor et al. 1990, 2004). Both in *A. fumigatus* and *A. nidulans*, null mutants of *acyA* and *cyaA*, respectively, render defects in conidial germination, conidial production, and vegetative hyphal growth due to the lowered intracellular levels of cAMP (Fillinger et al. 2002; Liebmann et al. 2003).

The *Aspergillus* genomes contain two catalytic subunits of PKA which are phylogenetically distant from each other. *pkaA* and *pkaC1*(Fig. 5.2) belong to group I PKAs, which are closely related to the three protein kinase A catalytic subunit (TPKs) homologs present in the genome of *S. cerevisiae* (Robertson and Fink 1998). In contrast, *pkaB* and *pkaC2* belong to the group II PKAs which are exclusively present in filamentous fungi (Ni et al. 2005; Fuller et al. 2011). In *A. nidulans,* the overexpression of *pkaA* causes reduced but not completely absent conidia production. In contrast, deletion of *A. nidulans pkaA* caused a hyperconidiating phenotype and limited radial growth (Shimizu and Keller 2001) indicating a role in vegetative growth. In addition, *pkaA* is a negative regulator (at both transcriptional and posttrancriptional level) for production of the carcinogenic secondary metabolite sterigmatocystin (Shimizu and Keller 2001). Curiously in *A. fumigatus* the deletion of the *pkaC1* gene resulted in a contrasting condition of low conidia formation but the same phenotypes of impaired germination and vegetative growth (Liebmann et al. 2004).

Likewise in other fungal species such as fission and budding yeast, *C. albicans,* and the filamentous saprophyte *Neurospora crassa*, there is only one adenylate cyclase in the genome of *Aspergillus*. Deletions of this gene in *A. nidulans* and *A. fumigatus* are not lethal, indicating that even in the absence of cAMP generated by the activity of CyaA or AcyA, the level of PKA activity is sufficient to signal for germination, hyphal growth, and conidiogenesis (Yu and d'Enfert 2008). In contrast, the loss of function of both genes encoding the two catalytic subunits of PKA, i. e., Δ*pkaA*/Δ*pkaB,* is lethal in *A. nidulans* (Ni et al. 2005). Remarkably, the same is not true for *A. fumigatus* where the double mutant strain Δ*pkaC1*/Δ*pkaC2* is viable although completely avirulent (Fuller et al. 2011).

The function of the single PKA regulatory subunit (*pkaR*) present in the genome of *A. fumigatus* and *A. niger* has been described, but a deletion mutant of this gene (AN4987) in the model organism *A. nidulans* has not been reported. In *A. fumigatus* the Δ*pkaR* strain resulted in impaired germination, vegetative growth, and conidia production as well as conidia hypersensitivity to oxidative stress agents (Zhao et al. 2006; Fuller et al. 2009). In the industrially important species *A. niger*, loss of function of the catalytic subunit of PKA (*pkaC*) resulted in a reduction of colony diameter, but the most severe phenotypes were, interestingly, observed in the PKA regulatory subunit (*pkaR*) disruption strain which resulted in very small colonies, absence of sporulation, and complete loss of growth polarity during submerged growth (Saudohar et al. 2002).

The current information in *Aspergilli* about GPCR and the cAMP-dependent protein A kinases (PKA) indicates that there is a striking diversity at the GPCR input level, but little diversity of components at the G-protein and cAMP-signaling level which is minimally conserved among the studied species but with some particularities. This may reflect the abilities of these fungi to adapt to various ecological niches and to integrate diverse environmental cues into highly conserved cellular processes (Lafon et al. 2006).

5.4.2 The Mitogen-Activated Protein Kinase Pathway

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine protein kinases, which are involved in the transduction of a variety of extracellular signals, regulating growth and differentiation processes. For this reason, MAPKs are central elements of several signaling pathways in eukaryotes. In addition, the MAPK mechanism of action involves the sequential phosphorylation of downstream protein targets of the MAP kinase pathway. Protein phosphorylation is regarded as one of the most important posttranslational modifications found in all eukaryotic organisms (Mok et al. 2011). The essential organization of the MAP kinase pathway consists of a sequential cascade of three protein kinases. On the top of the cascade, there is a MAPKKK (MAP kinase kinase kinase) which functions to phosphorylate the residues of serine and threonine of the immediately downstream MAKK (MAP kinase kinase), the second component of the pathway, which

Table 5.2 Components of the MAP kinase cascades in *Aspergillus* species

	Aspergillus	*S. cerevisiae* counterpart
Cell wall integrity pathway		
MAPKKK	*bckA/bck1*	BCK1
MAPKK	*mkkA/mkk2*	MKK1
MAPK	*mpkA*	MPK1 (SLT2)
High-osmolarity glycerol pathway		
MAPKKK	*sskB*	SSK2
MAPKK	*pbsA* or *pbsB/pbsB*	PBS2
MAPK	*hogA/sakA*	HOG1
Mating pathway		
MAPKKK	*steC*	STE11
MAPKK	*ste7/mkkB*	STE7
MAPK	*fus3/mpkB*	FUS3
mpkC pathway		
MAPKKK	–	–
MAPKK	–	–
MAPK	*mpkC*	–

Additional information about each gene can be found at the *Aspergillu*s Genome Database (AspGD) available at <http://www.aspergillusgenome.org/> (Arnaud et al. 2012) using the gene name provided here as entries. Gene names separated by a slash indicate aliases in the same *Aspergillus* specie and/or between them

becomes in turn activated. The activated MAPKK in a sequential manner dually phosphorylates the last component of the pathway, MAPK.

The signal that leads to activation of the MAPK cascade is perceived by a variety of types of receptors: G-protein-coupled (discussed in the previous section), histidine kinases phosphorelay sensors (described in the next section), and integral membrane proteins (Banuett 1998). After MAP kinase activation, the final target of the pathway is, in general, a transcription factor whose phosphorylation and activation enables the transcription of target genes to adjust the cell to the condition sensed by the receptor (Hill and Treisman 1995; Treisman 1996). There are four MAP kinases in the genome of *A. nidulans*, *A. fumigatus,* and *A. oryzae*, i.e., four different kinases that phosphorylate different transcription factors to adapt the cell to the extracellular stimuli (Reyes et al. 2006; Zhao et al. 2007; May 2008). These genes are named *mpkA*, *mpkB*, *sakA/HogA*, and *mpkC*. The first three are homologs of the yeast Slt2, Fus3, and Hog1, respectively, while *mpkC* has no orthologous genes or a pathway in *S. cerevisiae*. Table 5.2 shows the MAP kinases identified in *A. nidulans* and *A. fumigatus* because these three species cover the majority of the functional characterization of the MAP kinase-signaling cascade which allows us to infer the function of such components in *Aspergillus* genomes.

The *Aspergillus* MAP kinase *mpkC* is the least characterized. Its sequence is very similar to *sakA/hogA* (Reyes et al. 2006). However, *mpkC* cannot be attributed to a specific cascade with a well-established cellular function such as the cell wall integrity (CWI) or the high-osmolarity glycerol pathways (HOG), which mainly respond to cell wall or osmotic stress input, respectively. Moreover, it is currently

unknown which MAPKKK and MAPKK signal leads to the downstream activation of *mpkC* (May 2008). In *A. fumigatus* and *A. nidulans*, Δ*mpkC* deletion strain showed a wild-type phenotype in minimal and complete medium under various concentrations of salts and solutes (Reyes et al. 2006; Jun et al. 2011). Nevertheless, it revealed an unexpected role in the assimilation of polyalcohol sugars, indicating that *mpkC* is involved in the signaling for carbon source utilization only in *A. fumigatus* (Reyes et al. 2006). Even though there is sequence similarity between *A. fumigatus mpkC* and *sakA*/*hogA* MAP kinases (68 % amino acid identity) (May et al. 2005), the transcriptional regulation of these two MAPK upon osmotic and oxidative stress or in response to carbon source shift indicated no overlapping patterns, suggesting that they may govern independent signaling pathways (Reyes et al. 2006). The same results were observed in the analysis of the Δ*mpkC* in *A. nidulans* which depicted no visible phenotype under standard growth conditions. However, a cross talk with the MAPK *hogA* was verified since the MAPKK *pbsA* was able to phosphorylate *mpkC* as well as its known target *hogA* in an overexpressing *mpkC* strain of *A. nidulans* (Furukawa et al. 2005).

One of the most studied MAP kinase pathways is the CWI which underlines the function and importance of the gene *mpkA* in *Aspergillus*. Deletion strains for almost all of the MAP kinase pathway components in the cell wall integrity (CWI) have been generated at least in *A. nidulans* and *A. fumigatus,* and results were sometimes corroborative in both organisms. In *A. nidulans,* the deletion of *mpkA* showed abnormal hyphal structure and vegetative growth defects which could be rescued in the presence of osmotic stabilizers (Bussink and Osmani 1999), as well as increased sensitivity to the β 1,3-glucan synthase inhibitor Caspofungin (Mircus et al. 2009). In *A. fumigatus*, the CWI genes *bck1*, *mkk2,* and *mpkA* (Table 5.2) have been associated with impaired filamentous growth, decreased resistance to cell wall-disturbing and oxidative-stressing agents, thermotolerance defects, and abnormal hyphal morphology (Valiante et al. 2008, 2009). However, *A. fumigatus mpkA* is dispensable for virulence in a mouse model for invasive aspergillosis (Valiante et al. 2008), while the *mkk2* promotes virulence attenuation in the same model (Du et al. 2002). Interestingly, all the components of the CWI MAP kinase circuit in *A. fumigatus* are involved in the production of the melanin-related pigment derived from tyrosine degradation, pyomelanin (Valiante et al. 2009).

In *A. oryzae*, the function of the *mpkA* in the CWI was observed in a study where the subtilisin-like-processing enzyme *hexB* was deleted (Mizutani et al. 2004). The *hexB* loss-of-function mutant showed constitutively upregulated levels of both *mpkA* transcripts and the phosphorylation levels of MpkA in *A. oryzae*. This indicates that the phenotypic traits observed in the *hexB* mutant could be attributed to disordered CWI signaling.

The circuit coordinated by the *mpkB* MAP kinase homolog in *S. cerevisiae* (Ste11-Ste7-Fus3) is known as the sexual pathway of the budding yeast since it responds to pheromones, induces differentiation processes, and triggers sexual mating of yeast (Bardwell 2005). The most studied gene in this pathway in *Aspergillus* is MAPKKK *steC* (Table 5.2). A *steC* deletion strain exhibited reduced growth, brown-pigmented mycelium, and aberrant-sized conidiophores.

Furthermore, Δ*steC* was unable to form cleistothecia under sexual development conditions (Wei et al. 2003). More recently, some studies have devoted attention to the deletion strain of the MAP kinase *mpkB*. Deletion of *A. nidulans mpkB* caused slow vegetative growth, aberrant hyphal morphology, and also no cleistothecia formation under several sexual-inducing conditions, indicating that *mpkB* has an essential role during successful sexual reproduction in *A. nidulans* (Jun et al. 2011). In addition, the Δ*mpkB* mutant exhibited a decrease in sterigmatocystin gene expression and low mycotoxin levels. The mutation also affected the expression of genes involved in penicillin and terrequinone A synthesis (Atoui et al. 2008). *mpkB* was also necessary for normal expression of *laeA* (a methyltransferase-domain protein), which has been found to regulate secondary metabolism gene clusters (Bok and Keller 2004; Atoui et al. 2008; Bayram et al. 2008, 2012; Bayram and Braus 2012).

5.4.3 Two-Component Histidine Kinase Phosphorelay System

The two-component histidine kinase (HK) phosphorelay system (or tcs) is a signaling pathway initially identified and characterized in bacteria. In eukaryotes it is present only in slime mold, plant, and fungal cells. This system enables these cell types to sense and react to the extracellular stimuli with intracellular responses since it is connected to an output component such as activation of transcription or of a MAP kinase-signaling cascade (Hoch 2000; Santos and Shiozaki 2001, 2004; Catlett et al. 2003). As a consequence, several processes such as differentiation, growth, osmosensing, and chemotaxis can be controlled by this signaling machinery (Hoch 2000). The mechanisms and proteins involved in this two-component phosphorelay system have received attention in fungi since no similar pathway has been described in animal cells. In contrast, the other regulatory networks such as MAP kinase and GPCR (discussed above) are also present in higher eukaryotes. Therefore, it could be a promising target for the development of new antifungal compounds (Santos and Shiozaki 2001; Bahn 2008; Li et al. 2010).

The architecture of HK in fungal cells differs from that in prokaryotes. In bacteria, HK is composed of a sensor histidine kinase (HK) and a separate protein called response regulator (RR). In response to external stimuli, the sensor HK is autophosphorylated at the histidine residue followed by the transfer of this phosphoryl group to a conserved aspartate residue within a receiver domain of its cognate RR protein [for reviews see Hoch (2000), Santos and Shiozaki (2001), Catlett et al. (2003), and Li et al. (2010)]. In contrast, and although there are some exceptions, fungal cells have a so-called hybrid HK which combines both functions of sensor HK and RR in the same protein (Li et al. 2010). In other words, the phosphoryl group from the sensor HK is transferred directly to the RR domain in the same polypeptide (Catlett et al. 2003; McCormick et al. 2012). Fig. 5.3 depicts

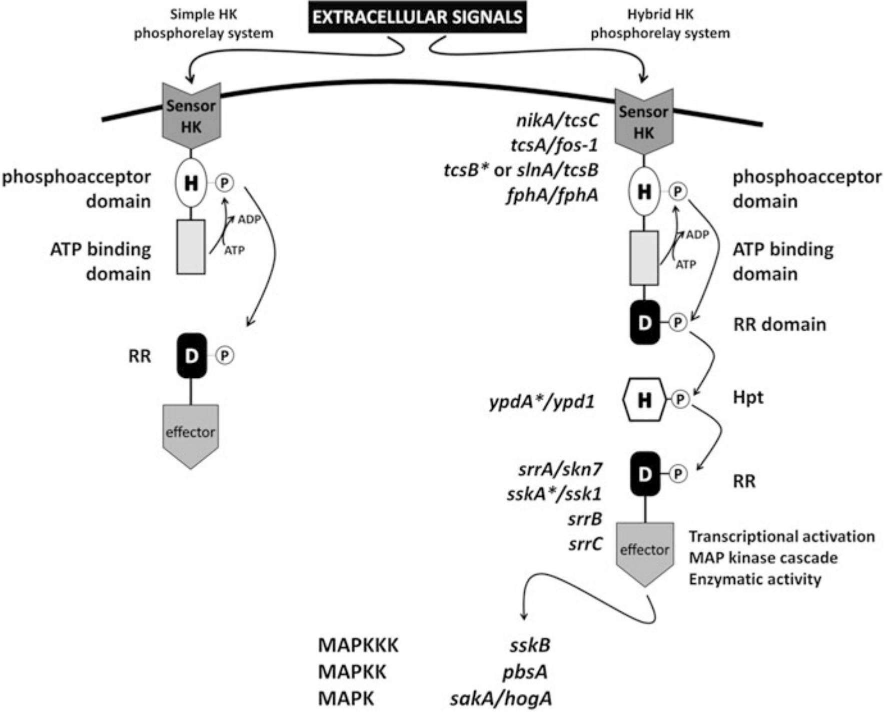

Fig. 5.3 Schematic representation and domain organization of the two-component histidine kinase (HK) phosphorelay system (or tcs) appearing in prokaryotes (*left*) or eukaryotes (*right*). Most eukaryotic and all fungal HKs are hybrids. An external stimulus is received by the sensor domain of the HK localized in the cell membrane. This signal typically catalyzes an ATP-dependent autophosphorylation reaction in which the protein kinase covalently attaches the γ-phosphoryl group from ATP to itself on a conserved histidine residue (indicated as **H**) in the phosphoacceptor domain. The phosphoryl group is subsequently transferred to a conserved aspartic acid residue (indicated as **D**) on a response regulator protein (**RR**) activating the effector domain to result in an output such as activation of transcription or a MAP kinase (MAPK) cascade or a specific enzymatic activity required. Hence, for a simple two-component system (*left*), the HK (sensor, phosphoacceptor, and ATP-binding domains) and the RR are separate proteins. On the other hand, for a hybrid two-component system (*right*), the HK (sensor, phosphoacceptor, and ATP-binding domains) and the second RR/effector protein are separate polypeptides. Structurally, a hybrid HK protein presents both HK and RR domains in the same polypeptide but generally requires additional rounds of phosphorelay events through an **HPt** domain (histidine-containing phosphotransfer intermediate protein, indicated as the *hexagon*) and a second cognate **RR** protein. The HPt domain can be part of the hybrid HK protein or a separate protein. The main components of the two-component phosphorelay system identified and characterized in *A. nidulans* and *A. fumigatus* are described. From the 15 putative HK sensors identified in these species, four of them are characterized. There is a single Hpt protein in *Aspergillus* (*ypdA*). The four putative cognate RR in these species have also been described. The gene names separated by a slash indicate the names in *A. nidulans* and *A. fumigatus*, respectively. The genes marked with an *asterisk* indicate those which belong to the circuit connecting the HK and HOG pathways in *A. nidulans* (for details see the text). In this circuit, the RR *sskA* activates the MAPKKK *sskB* ultimately activating the HOG pathway to drive the cell adaptation in conditions of hyperosmotic shock. Adapted from West and Stock (2001), Catlett et al. (2003), and Capra and Laub (2012)

the main differences in the prokaryote and eukaryote systems and highlights main events on the hybrid HK-mediated signal transduction cascade.

The fungal phosphorelay systems consist of a single or several hybrid HKs, a histidine-containing phosphotransfer (Hpt) protein, and one or two additional downstream canonical RRs (Vargas-Perez et al. 2007; McCormick et al. 2012). The Hpt component consists of a protein with a conserved motif (PFAM01627) where a second residue of histidine serves as a receptor for a second transfer of the phosphoryl group coming from the RR domain of the fungal hybrid HK (Fig. 5.3). This additional phosphorelay step may allow the organism to integrate multiple input signals arriving at the hybrid HK into a single output (Appleby et al. 1996; Catlett et al. 2003). The second RR component of the hybrid HK pathway mediates the activation of the effector, such as a MAP kinase cascade (Catlett et al. 2003).

Through analysis of *Aspergillus* and other filamentous fungal genomes, such as *Neurospora crassa*, *Fusarium verticillioides, Cochliobolus heterostrophus,* and *Botrytis cinerea*, 11 families of hybrid HK have been described in fungi, which were classified according to their protein sequence and domain organization [for reviews about HK families, see Catlett et al. (2003) and Hagiwara et al. (2007a)]. Compared to prokaryotes, yeasts such as *S. cerevisiae*, *S. pombe,* and *C. albicans* have a much lower number of hybrid HKs. For instance, in *S. cerevisiae* there is only one hybrid HK (Sln1), three RR (Ssk1, Skn7, and Rim15), and one Hpt (Ypd1) (Abe et al. 2010; Jung et al. 2012). Conversely, the genome of *A. nidulans* contains 15 hybrid HKs, four RR, and also a single one Hpt (Hagiwara et al. 2009). Interestingly, these hybrid HKs are more redundant than those identified in the essentially saprobic fungus *N. crassa*, which indicates the importance of the HK phosphorelay system to the *Aspergillus* species in the sensing and signaling of external stimuli (Abe et al. 2010).

Comparison of the *A. nidulans*, *A. oryzae,* and *A. fumigatus* genomes identified 15, 15, and 13 putative hybrid HKs, respectively, in these organisms. These HKs represent nine of the 11 families of HK described by Catlett et al. (2003)and Abe et al. (2010). Besides its functional relevance, the characterization of the hybrid HK in the *Aspergillus* species is still in its infancy compared to other families of signaling pathways. The best characterized HKs in *Aspergillus* are those in the model organism *A. nidulans* and in the opportunistic pathogen *A. fumigatus*. From the 15 HK identified in the genome of *A. nidulans* (Hagiwara et al. 2007a, 2009; Vargas-Perez et al. 2007), four have been characterized: *nikA*, *tcsA*, *tcsB* (or *slnA*, the homolog of *S. cerevisiae* Sln1), and *fphA* (Virginia et al. 2000; Furukawa et al. 2002; Blumenstein et al. 2005; Hagiwara et al. 2007b). Figure 5.3 shows the gene families currently characterized and its function in the *Aspergilli* phosphorelay system.

The *nikA* null mutant showed decreased radial growth but was not hypersensitive to high osmolarity (Vargas-Perez et al. 2007; Hagiwara et al. 2009). It was also required for efficient conidia production and expression of the transcription factor BrlA in *Aspergillus* involved in the early stages of asexual reproduction, and conidiogenesis was considerably lower in the Δ*nikA* strain (Hagiwara et al. 2009). The *tcsA* disruption strain in *A. nidulans* showed a complimentary phenotype with

the other HKs since it was also required for production of conidia but not for vegetative growth (Virginia et al. 2000).

The other characterized HK in *A. nidulans*, *tcsB* (or *slnA*), presented no detectable phenotype regarding vegetative growth or conidiation or in response to various stimuli such as osmotic and oxidative stressing agents and antifungal drugs (Furukawa et al. 2002, 2005). However, in the model yeast *S. cerevisiae,* there are strong evidences that the SLN1 (the homolog of *A. nidulans tcsB*) directly senses osmotic changes providing a connection between the HOG MAP kinase pathway and the HK pathway (Hohmann 2002). In yeast, the sensor Sln1p transfers the phosphate from its receiver domain to the Ypd1p under low-osmolarity conditions. Nevertheless, this phosphoryl transfer activity is reduced when the cell senses an osmotic shift to hyperosmotic condition. This scenario indicates that under hyposmolarity conditions, the HK is active and continuously phosphorylates the Hpt protein Ypd1p. Ypd1 phosphotransfer signal transducer therefore negatively regulates Ssk1p, thus preventing signaling beyond this step in the cascade. On the other hand, upon a hyperosmotic shock, the HK activity of Sln1p drops transiently, removing the negative regulation of Ssk1 eventually leading to activation of the downstream MAPKKK Ssk2p (Table 5.1) (Hohmann 2002).

The same hypertonic stress response is thought to occur in *A. nidulans* in that the loss of the negative regulation of the RR *sskA* (homolog of *S. cerevisiae* SSK1) results in phosphorylation of MAPKKK *sskB,* followed by MAPKK *pbsA* and consequent activation of the *sakA*/*hogA* MAPK pathway (Table 5.2 and Fig. 5.3). Interestingly, in *A. nidulans* the deletion of *tcsB* alone does not cause sensitivity to osmotic stress, which indicates that other HK may act in a compensatory manner and that *A. nidulans* has a more complex and robust osmoregulatory system than the yeast SLN1-YPD1-SSK1-HOG1 MAPK cascade (Furukawa et al. 2002, 2005; May 2008). The *A. fumigatus tcsB* counterpart is involved in partial tolerance to SDS, but as in *A. nidulans*, no apparent growth differences were observed under various growth conditions and stress induction. However, even with reports of minimal phenotypes "in vitro," the expression of *tcsB* (initially described as AfHK1) was increased in fungal cells isolated directly from the infected host (Du et al. 2002).

Functional descriptions are also available for *fos-1* (the homolog of *A. nidulans tcsA*), *tcsB* (*A. nidulans tcsB*), and *tcsC* (the homolog of *A. nidulans nikA*) in the pathogen *A. fumigatus* (Pott et al. 2000; Du et al. 2002, 2006; McCormick et al. 2012). Deletion of *A. fumigatus fos-1* greatly attenuated the virulence (Clemons et al. 2002) although the deletion mutant showed only minimal phenotypic changes in liquid or solid cultures. Only in liquid medium there was a delay in conidiophore development and strong resistance to the antifungal agents dicarboximide and cell wall-degrading enzymes, thus indicating a regulatory role of *fos-1* in cell wall organization (Pott et al. 2000). The HK *tcsC* of *A. fumigatus* is required for growth under hyperosmotic stress, hypoxia, and farnesol exposure but dispensable for normal growth, sporulation, and conidial viability. A characteristic feature of the Δ*tcsC* mutant is its resistance to certain fungicides, like fludioxonil. Both hyperosmotic stress and treatment with fludioxonil result in a TcsC-dependent

phosphorylation of the MAP kinase SakA, confirming a role for TcsC in this signaling pathway. However, *tcsC* is dispensable for virulence in *A. fumigatus* (McCormick et al. 2012).

The phytochrome *fphA* gene still remains uncharacterized in *A. fumigatus*. In *A. oryzae*, the only HK partially described is the *fphA* homolog which is known to be continuously expressed under red-light conditions (Hatakeyama et al. 2007). In *A. nidulans fphA* functions as a red-light sensor, and deletion of *fphA* leads to partial derepression of sexual development under red-light conditions (Blumenstein et al. 2005). In *A. niger* although the same orthologs of HK are present, with the exception of *tcsB*, none of them has been characterized yet.

The four RR in *A. nidulans* have also been functionally characterized (Hagiwara et al. 2007a; Vargas-Perez et al. 2007). The deletion strains Δ*ssrB* and Δ*ssrC* showed no phenotypes under the conditions tested. In contrast, *A. nidulans ssrA* and *sskA* are involved in osmotic stress resistance and are also required for oxidative stress tolerance (Hagiwara et al. 2007a; Vargas-Perez et al. 2007). In *A. fumigatus* the deletion mutant of *skn7* (*S. cerevisiae* SKN7) was morphologically similar to the wild-type strain but showed a decreased resistance to hydrogen peroxide and t-butyl hydroperoxide indicating the role of this gene in the detoxification of reactive oxygen species (Lamarre et al. 2007). It was also dispensable for virulence. Interestingly, the histidine-containing phosphotransfer intermediate protein, (Hpt) gene, *ypdA* is essential in *A. nidulans* (Furukawa et al. 2005; Vargas-Perez et al. 2007). This essentiality is due to the fact that the deletion of *ypdA* constitutively activates the HOG pathway by the reasons mentioned above.

Collectively these data indicate that the genome sequences of *Aspergillus* have fostered the study of the HKs present in such organisms as an important component of the signal transduction pathways. Depending on the species, the HK phosphorelay system can be implicated in the response to external stimuli such as osmotic and oxidative, hypoxia, resistance to antifungal compounds, and sexual and vegetative development (Santos and Shiozaki 2001; Catlett et al. 2003; Vargas-Perez et al. 2007; Bahn 2008; McCormick et al. 2012). Also, signaling pathways mediated by hybrid HK in *Aspergillus* differ in some aspects from those in *S. cerevisiae* or the pathogenic yeast *C. albicans* in terms of phenotypic traits since different responses to stressing conditions were identified (Du et al. 2006).

5.5 Secondary Metabolites of Aspergilli

The secondary metabolites of *Aspergillus* species impact our lives on a daily basis and span the range from harmful mycotoxins to beneficial pharmaceuticals, commodity chemical intermediates, and food additives. In addition, they are useful markers for the polyphasic approach to *Aspergillus* taxonomy [reviewed in Frisvad et al. (2008)]. The biosynthetic genes for secondary metabolites are often clustered. It is clear from the genomes of the *Aspergillus* species sequenced thus far that the molecules that have been characterized currently constitute a small percentage of

the potential production of secondary metabolites by *Aspergillus* and other ascomycetes. Progress in associating genetic pathways with production of specific secondary metabolites has been most recently reviewed by Sanchez et al. (2012). Advances in understanding regulation of secondary metabolite biosynthesis have also facilitated the detection of "silent" gene clusters. A combination of pathway-specific regulators working both in cis and trans with gene clusters as well as global regulators are responsible for activation and repression of secondary metabolite biosynthesis [reviewed by Brakhage (2013)]. In this section, we present a high-level overview of some of the important secondary metabolites derived from species of *Aspergillus* and for which a gene cluster is known and discuss current approaches for secondary metabolite analysis that take advantage of *Aspergillus* genomic resources.

5.5.1 Aflatoxin

In 1960, Turkey "X" Disease afflicted poultry in Great Britain. The course of the disease was "...generally short, and once a poultry was seen to be affected it would usually die within a week" (Bount 1961). The cause of the disease was quickly traced to *A. flavus*-contaminated peanut-derived feed imported from South America (Nesbitt et al. 1962; Van der Zijden et al. 1962). Subsequently members of the aflatoxin family of compounds were purified and the structures determined (Asao et al. 1963). Over the following decades a variety of approaches were used to elucidate the biosynthetic pathway of aflatoxin using a host of *Aspergillus* species. Gene clusters for production of aflatoxin or sterigmatocystin (a less toxic compound found late in the aflatoxin pathway and the final product of *Aspergillus nidulans*) have been elucidated (Yu et al. 2004b). Aflatoxin and sterigmatocystin are produced by the action of a polyketide synthase (PKS) and several modifying enzymes (Yu et al. 1995, 2004a; Brown et al. 1996b; Cary et al. 2009). The "starter unit" for aflatoxin is hexanoic acid, which is produced by the action a fatty acid synthase encoded by two genes in the cluster (Brown et al. 1996a; Watanabe et al. 1996). Interestingly, what appear to be nonfunctional versions of the aflatoxin biosynthetic pathway are found in the genomes of *A. oryzae* and *A. sojae*, two fungi commonly used in koji mold processes (Watson et al. 1999; Matsushima et al. 2001a, b; Takahashi et al. 2002; Lee et al. 2006; Tominaga et al. 2006; Kiyota et al. 2011).

5.5.2 Ochratoxin

Discovered in 1965 (van der Merwe et al. 1965), ochratoxin A (OTA) is an important contaminant of grains, meat, and wine. It is produced by a number of *Aspergillus* species including *A. carbonarius* and some strains of *Aspergillus niger* (Nielsen et al. 2009; Frisvad et al. 2011), as well as some species of *Penicillium*

(van Walbeek et al. 1969; Pitt 1987). A putative gene cluster elucidated through the genome sequencing of *A. niger* strain CBS 513.88 (Pel et al. 2007) supported the existing hypothesis that the biosynthetic pathway involves both a PKS and a non-ribosomal peptide synthetase (NRPS) (O'Callaghan and Dobson 2006). There is a large deletion within this cluster found in the *A. niger* strain ATCC 1015—a non-OTA-producing strain—genome sequence (Andersen et al. 2011). Orthologs of the putative *A. niger* OTA biosynthetic genes were identified in the genome of *A. carbonarius* ITEM 5010, and the NRPS was subsequently deleted. The strain containing the deletion was unable to produce OTA, confirming the identification of the *A. carbonarius* OTA biosynthetic cluster (Gallo et al. 2012).

5.5.3 Fumonisins

Fumonisins are mycotoxins that have been well described in the ascomycete genus *Fusarium,* having been initially characterized in the late 1980s (Bezuidenhout et al. 1988; Gelderblom et al. 1988). It was therefore somewhat of a surprise when clusters encoding orthologs of genes whose products are involved in the fumonisin biosynthetic pathway were discovered in two *A. niger* strains whose genomes had been sequenced, ATCC 1015 and CBS 513.88 (Baker 2006; Pel et al. 2007). The production of fumonisin was later confirmed biochemically (Frisvad et al. 2007).

5.5.4 Lovastatin

The statins have had an enormous impact on the health of millions. Akira Endo, a researcher at Japan-based Sankyo, is credited with the discovery of a class of compounds (compactins from *Penicillium citrinum*) that inhibited HMG coA reductase, a key enzyme in cholesterol biosynthesis (Endo et al. 1976a, b, 1977). In the late 1970s researchers at Merck isolated mevinolin (lovastatin) from *A. terreus* (Alberts et al. 1980). By the late 1980s lovastatin had been approved as a treatment for high cholesterol in the United States [see this concise history of the statins (Tobert 2003)]. Lovastatin is a polyketide whose biosynthetic pathway has been elucidated—the gene cluster involved in lovastatin biosynthesis encodes two polyketide synthases as well as regulatory and numerous backbone "decorating" enzymes (Hendrickson et al. 1999; Kennedy et al. 1999).

5.5.5 *Associating Secondary Metabolites with Biosynthetic Pathways*

One striking result from the early *Aspergillus* sequencing projects was the diversity of secondary metabolite biosynthetic genes encoded within each genome (Galagan et al. 2005; Machida et al. 2005). The diversity of the genes encoding secondary metabolite biosynthetic genes has grown with each Aspergillus genome sequenced (Pel et al. 2007; Andersen et al. 2011). This vast catalog of polyketide synthases, terpene synthases, non-ribosomal peptide synthetases, and fatty acid synthases represents an uncharacterized frontier of chemical diversity whose exploration has been enabled by genome sequencing. The work of Kroken et al. (2003) is an excellent example of a phylogenetic approach that was used to characterize polyketide synthases. Similar analyses of secondary metabolite diversity have been performed utilizing one or more *Aspergillus* genomes [see, for example, Varga et al. (2003), Cramer et al. (2006), and Ferracin et al. (2012)].

There are a number of molecular genetic strategies for associating metabolites with their respective biosynthetic genes (Sanchez et al. 2012). A computational tool for identification of secondary metabolite gene clusters from *Aspergillus* genomic sequences is SMURF (Secondary Metabolite Unknown Regions Finder) (Khaldi et al. 2010; Sanchez et al. 2012). "Classical" genetic analysis combined with genomic sequence information has also been successful in associating pigment production with *A. niger* pigment biosynthetic genes (Jorgensen et al. 2011). Another approach to characterizing pigment production in *A. niger* was to identify orthologs of pigment pathways characterized in other fungal species (Baker 2008; Chiang et al. 2011). Combining genomics with molecular genetic manipulation of individual genes, whether through overexpression, deletion, or heterologous expression, has greatly accelerated the pace of associating genes and gene clusters with their respective secondary metabolites and biosynthetic pathways [reviewed in Sanchez et al. (2012)].

5.6 Concluding Remarks

This chapter has summarized and given only some examples of the impact the availability of genome sequences has had on *Aspergillus* research. It was predicted previously that sequencing and annotation of the first three Aspergilli will be seen as a transformational event in *Aspergillus* biology (Nierman et al. 2005a), and this has certainly come through. Soon more than 50 genomes of Aspergilli and related species will be available, providing a resource for both in-depth and comparative studies on a variety of topics. The combination of a large community that addresses nearly every aspect of *Aspergillus* biology; the medical, agricultural, and biotechnological relevance of the genus; and the development of high-quality tools and

infrastructures for detailed (post-)genomic analysis will guarantee that *Aspergillus* will remain at the forefront of fungal research, also in the field of genomics.

Acknowledgments GHG and IM were supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Chapter 6
Trichoderma: Genomic Aspects of Mycoparasitism and Biomass Degradation

Christian P. Kubicek and Irina S. Druzhinina

6.1 Introduction

Species of *Trichoderma* (teleomorph *Hypocrea*,[1] Hypocreales, Ascomycota, Dikarya) have been isolated from an innumerable diversity of natural and artificial substrates, thus demonstrating their high opportunistic potential and adaptability to various ecological conditions (Druzhinina et al. 2011). *Trichoderma* has a broad impact on mankind: one of its species—*T*. reesei—has become the major industrial producer of enzymes for conversion of plant biomass into soluble sugars for biofuel and biorefinery production (Kumar et al. 2008; Kubicek et al. 2009). In addition, the intrinsic ability of *Trichoderma* to parasitize or even prey on other fungi has been widely used for the biological control of pests (mainly against fungi, but also against nematodes), particularly with strains of *T*. cf. *harzianum*, *T*. *atroviride*, *T*. *virens*, and *T*. *asperellum* (Benitez et al. 2004; Harman et al. 2004; Druzhinina

[1] In this review we accommodate the changes proposed at the International Botanical Congress in July 2011 for the International Code of Botanical Nomenclature and the ongoing discussion on the future single taxon name for *Hypocrea/Trichoderma* that may be followed at the website of the IUMS International Subcommission on *Trichoderma* taxonomy at http://www.isth.info/content.php?page_id=102. Therefore we use the single generic name *Trichoderma* not only for asexual species but also for holomorphs when the sexual stage is described. However at first mention of holomorphic species both teleomorph (*Hypocrea*) and anamorph (*Trichoderma*) names are given. When the whole genus of *Trichoderma* and *Hypocrea* spp. is considered, the term *Trichoderma* is applied.

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*,
Soil Biology 36, DOI 10.1007/978-3-642-39339-6_6,
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et al. 2011). More recently, this ability was expanded by the findings that some *Trichoderma* spp. can trigger and enhance the self-defense of plants towards pathogens as well (Aly et al. 2011; Shoresh et al. 2010).

Yet some species of the genus *Trichoderma* also have negative impacts on mankind. In a clinical context, some species (mainly *T. longibrachiatum* and *Hypocrea orientalis*) have been shown to occur as opportunistic pathogens of immunocompromised humans (Kredics et al. 2011). In addition, two species (*T. brevicompactum* and *T. arundinaceum*) were proven to be the only trichothecene producer within *Trichoderma* (Nielsen et al. 2005). Plant pathogenic strains of *Trichoderma* have not been reported, but a small number of species (*T. aggressivum*, *T. pleuroticola*, *T. pleurotum*, and *T. mienum*) have been found as mycoparasites in the commercial mushrooms *Agaricus* and *Pleurotus*, respectively (Seaby 1998; Samuels et al. 2002; Komoń-Zelazowska et al. 2007; Kim et al. 2012).

All these properties make *Trichoderma* an interesting object for comparative genomics, which may lead to the identification of genes and pathways responsible for this panoply of interactions with the environment. Before discussing the results that have been obtained so far, we shall briefly describe the biology of these fungi.

6.2 Biology of *Trichoderma*

6.2.1 Taxonomy and Phylogeny of Trichoderma

The genus *Trichoderma* was established by Persoon (1794) based on four species collected in Germany (see Samuels 2006). Of these taxa only one species, *T. viride*, remained in *Trichoderma*. Bisby (1939) postulated that *Trichoderma* consists only of a single species, and this concept remained valid until the end of the 1960s. Therefore, all *Trichoderma* strains that were isolated in this time were named *T. viride*—a fact that led to a confusion about species producing enzymes, mycotoxins, and acting as mycoparasites in the literature and even in textbooks. In 1969, Rifai (1969) fundamentally revised the species concept of *Trichoderma* by recognizing nine aggregate species and postulated that each of these aggregates may actually turn out to be several species once appropriate methods for distinction would become available. His work was expanded and revised by Bissett (1984, 1991a, b, c, 1992), who grouped Rifai's aggregate species into the five sections, each of which consisted of several species.

In the late nineties, PCR and DNA sequence analysis were introduced and became widely accepted as a standard technique in fungal systematics. Application of these tools and the development of appropriate theoretical frameworks for the use of molecular analytical data in species recognition rapidly led to an increase in the number of species in *Trichoderma* (Druzhinina et al. 2006). Today, the application of the genealogic concordance phylogenetic species recognition concept

(Taylor et al. 2000) is the gold standard of molecular species identification also in *Trichoderma* and has led to a reappraisal of the phylogeny of all the sections proposed by Bissett. Although some sections were proven to be monophyletic, most other sections showed paraphyly. Consequently, molecular taxonomists prefer to talk of *Trichoderma* "clades" in the sense of strains that emerge from statistically supported hypothetical taxonomic units (nodes) or lone lineages. As of the time of this writing, *Trichoderma* consists of at least 200 species that are contained in 16 phylogenetic clades and several lone lineages [for a detailed review, see Atanasova et al. (2013a)].

6.2.2 *Developmental Biology of* Trichoderma

Trichoderma spp. have a heterothallic mode of sexual reproduction. Consequently, mating is only possible between individuals that contain different mating types genes, *mat1-1* and *mat1-2*, which reside in the same chromosomal location but lack sequence similarity. This "bipolar heterothallism" has recently been genetically characterized in *T. reesei* (Seidl et al. 2008), but its occurrence is also evident in *T. virens* and *T. atroviride* from the findings of only one of the two mating types in the genomes of the respective strains.

Saprotrophic growth of *Trichoderma* is usually accompanied by the production of asexual spores. Sporulation depends primarily on the type and availability of the carbon source (Friedl et al. 2008) and can be modulated by other environmental parameters such as illumination, nutrient deprivation, low pH, and mechanical injury (Steyaert et al. 2010; Schmoll et al. 2010; Carreras-Villasenor et al. 2012). Interestingly, sporulation in *Trichoderma* can also be induced by volatile organic compounds (mainly medium chain alcohols) from neighboring colonies of the same species (Nemcovic et al. 2008) or by water soluble metabolites (Friedl and Druzhinina, 2012), suggesting the existence of a sort of *quorum sensing* mechanism.

Results from field observations in Central Europe suggest that the *Trichoderma* anamorphs usually develop before the teleomorph, but sometimes conidiophores are also found on overmature stromata, illustrating the broader range of optimal environmental conditions for anamorph compared to teleomorph (Jaklitsch 2009, 2011).

Molecular strain taxonomy has led to the identification of many teleomorph-anamorph relationships in *Trichoderma*, but true clonality has so far been confirmed for several species using *in silico* population analytic methods (e.g., *T. longibrachiatum*, *T. parareesei*, *T. harzianum* sensu stricto (Druzhinina et al. 2008; Druzhinina et al. 2010a, b; Atanasova et al. 2010) and proposed for *T. asperellum* and *T. asperelloides*; Samuels et al. 2010). Interestingly, all confirmed clonal species of *Trichoderma* appear to be cosmopolitan (at least for a certain climatic range such as the tropics or temperate climates), suggesting a link between intense asexual reproduction and environmental opportunism.

6.2.3 Trichoderma *and Its Habitats*

A rather limited group of *Trichoderma* species, all being successful opportunists, can be found in a wide range of habitats. They have consequently been isolated from water, soil, decaying wood, walls, and even nonnatural materials such as rubber foam or kerosene tanks (Klein and Eveleigh 1998). As explained recently (Kubicek et al. 2011; Druzhinina et al. 2011), the innate nature of almost all *Trichoderma* spp. is mycoparasitism or mycotrophy, i.e., the attack and eventual killing of other fungi and feeding on them. The molecular physiology of this process has been studied to some extent and can be divided into several stages: recognition of the presence of a potential prey ("sensing"), induction of the biochemical tools to besiege the prey, actual attack, and eventual killing and feeding on the prey. In particular, the signaling between *Trichoderma* and the host and the role of extracellular hydrolytic enzymes and secondary metabolites have been studied intensively, and the results obtained are the subject of several reviews (Viterbo et al. 2002; Seidl 2008; Vinale et al. 2008; Omann and Zeilinger 2010; Susi et al. 2011; Mukherjee et al. 2012a, b; Druzhinina et al. 2012).

The second habitat that is frequently associated with some *Trichoderma* species is the rhizosphere of plants. *Trichoderma* spp. have been known since decades to be "rhizosphere competent," i.e., to grow and develop within the soil volume influenced by root exudates. This takes place without causing disease but eventually antagonizing pathogenic microorganisms (Lewis and Papavizas 1984). However, metagenomic analyses showed that *Trichoderma* spp. are rather infrequent in soils (Bue et al. 2009; Lim et al. 2010). It is therefore difficult to decide whether rhizosphere is in fact a natural habitat of *Trichoderma*, or if the fungus follows its preys/hosts into the soil in general and to the rhizosphere in particular [for review and arguments, see Druzhinina et al. (2011)]. However, in any case some *Trichoderma* spp. can trigger an induced systemic resistance (ISR) in the plants. This induction starts with the recognition of microbe-associated molecular patterns by so-called pattern recognition receptors of the plant, which subsequently activates a primary defense response in the plant. *Trichoderma* molecules that have been shown to trigger ISR include secreted xylanases, cellulases, and the cellulose-binding protein swolenin [see Shoresh et al. (2010)], small cysteine-rich secreted proteins (Djonovic et al. 2006, 2007), peptaibols (Viterbo et al. 2007; Leitgeb et al. 2007), and an unknown PKS-NRPS product (Mukherjee et al. 2012b). In all these cases, knockout of the respective genes did not impair the ability of *Trichoderma* to colonize the roots, although the induction of ISR was abolished in most cases. Thus, *Trichoderma*, at first glance, does not seem to benefit from the plant's response. There is a debate as to whether the interaction between *Trichoderma* and plants is a form of symbiosis (Harman et al. 2004). Based on available data, this hypothesis cannot be proven (Druzhinina et al. 2011). However, some *Trichoderma* taxa (including several novel species) are reported to live inside the plants as endophytes and thereby contribute to the health of the plants by promoting plant growth, delaying onset of drought stress and inhibition of

pathogens (Bae et al. 2009; Bailey et al. 2006; Hanada et al. 2008, 2010; Jaklitsch et al. 2006; Samuels et al. 2006; Tejesvi et al. 2006). Druzhinina et al. (2011) proposed that mycotrophs may have become endophytes by entering the plant roots via parasitism on mycorrhizal fungi as described by (de Jaeger et al. 2010).

The opportunistic nature of *Trichoderma* spp. has also allowed some species to undergo various types of interactions with other organisms, including marine sponges, terrestrial invertebrates, and mammals [for review, see Druzhinina et al. (2011)]. Some *Trichoderma* spp. can successfully antagonize and kill plant parasitic nematodes, offering a new not-yet-fully explored possibility to combat these agricultural pests (Casas-Flores and Herrera-Estrella 2007). *Trichoderma* recently also joined the emerging list of such opportunistic pathogenic fungi that cause invasive mycoses of mammals, including humans, under conditions of an impaired immune system (Kredics et al. 2011). So far mainly two species—*T. longibrachiatum* and *H*. orientalis—have been proven to infect immunocompromised patients. The mechanisms of these interactions have not been studied yet, but the enhanced arsenal of proteases (as described above) may play an important role in this trait [for review, see Druzhinina et al. (2011)].

6.3 Genomic Attributes of *Trichoderma*

6.3.1 General Genomic Features

The wild-type progenitor of the industrial cellulase producer strains of *T. reesei*, *T. reesei* QM 6a, was the first *Trichoderma* species whose genome was sequenced (Martinez et al. 2008). The rationale for this was that all *T. reesei* strains currently used in industry or by academics are mutants of this single wild-type isolate. Consequently, the genomes of several of these enhanced producers (QM 9123, QM 9414, NG14, RUT C30) were subsequently also sequenced. The sequence was compared to QM 6a in order to trace the genomic alterations that accompanied selection for increased cellulase production (Le Crom et al. 2009; Vitikainen et al. 2010; see Sect. 6.5, below). Three years later, the genomes of two *Trichoderma* spp. that are vigorous mycoparasites—*T. virens* Gv29-8 and *T. atroviride* IMI 206040—were also published (Kubicek et al. 2011). Today, the genomes of two more strong mycoparasites, *T. harzianum* sensu stricto (CBS 226.95) and *T. asperellum* (CBS 433.97), and the two facultative human pathogens, *T. longibrachiatum* (ATCC 18648) and *T. citrinoviride*, have also been sequenced and are publically available at JGI (http://www.genome.jgi-psf.org/), and more (e.g., *T. parareesei*, *T.* cf. *harzianum*, and *T. koningii*) were sequenced in proprietary projects in Europe and China. However, at the time of this writing, all of them are still in the process of analysis, and the preliminary data obtained so far will not be used in this review. This review will therefore concentrate on data from *T. reesei*, *T. atroviride*, and *T. virens* only.

Table 6.1 Chromosomes of *T. reesei* and genes mapped on them[a]

Chromosome	Size (mbp)	Mapped genes	Scaffolds	Size (mbp)
1	3.6	*pyr4*, *egl2*, *xyn1*	1	2.75
		cbh2	3	1.91
		benA	16	0.82
		xyn2	27	0.43
		cbh1	29	0.38
2	3.2	*pyr2*	2	2.00
3	4.2			
4	4.2			
5	5.1	*pma1*[a]	11	1.15
6	6	*tub1*	37	<0.2
7	6.2	*egl1*	10	1.15
		bgl1	6	1.45

[a]Data taken form Carter et al. (1992)

The genome sizes of *T. reesei*, *T. atroviride*, and *T. virens* are 34.1, 36.1, and 38.8 Mbp, respectively, and harbor 9143, 11865, and 12518 gene models, in the range of average numbers for Pezizomycotina fungi (see DOE JGI program MycoCosm http://www.genome.jgi.doe.gov/programs/fungi/index.jsf). All strains examined appear to have seven chromosomes. The sizes of the chromosomal bands in strain *T. reesei* QM 6a are approximately 6.2, 6.0, 5.1, 2 × 4.2 (doublet), 3.6, and 3.2 Mb (Carter et al. 1992; Mäntylä et al. 1992). Their cumulative size (32.5 Mbp) correlates with the 34.1 Mbps determined from genome sequencing. Herrera-Estrella et al. (1993) reported the presence of six chromosomes in *T. reesei* QM 6a and *T.* "*harzianum*" (which was later reidentified to actually be *T. atroviride*) IMI 206040, but this smaller number is likely the result of overlooking the doublet chromosomal band. No chromosome numbers are yet available for *T. virens*. Despite this information, genome mapping has not been performed yet because the ability to cross *Trichoderma* spp. was not available until recently (Seidl et al. 2008). The genome sequence of *T. reesei* is therefore still distributed among 71 scaffolds, of which the longest (scaffold 1) is 2.75 Mb and the shortest 8,513 bps. A number of genes have been mapped to the chromosomes (Table 6.1), but they do not yet allow the drawing of a draft map. In fact, the data suggest that the gene order on some of the scaffolds does not resemble that on the chromosome, because the sum of all scaffolds that contain genes that (Carter et al. 1992) mapped in chromosome 1 would far exceed the size of this chromosome (Table 6.1). Only a few genes have been mapped on the *T. atroviride* chromosomes (Herrera-Estrella et al. 1993).

The comparably smaller gene repertoire in *T. reesei* is likely due to the operation of the repeat-induced point mutation (RIP) effect (Selker 1990), because among these three species, *T. reesei* is the only one that is known almost exclusively from sexually propagating cultures (Kubicek et al. 2011). A similar small genome size was also detected for *T. citrinoviride* (unpublished data), which in temperate climates is also predominantly found in the form of its teleomorph *Hypocrea*

schweinitzii (Jaklitsch 2009), whereas the newly sequenced asexual species *T. longibrachiatum*, *T. harzianum* sensu stricto, and *T. asperellum* display genome sizes in the range of those found in *T. atroviride* and *T. virens* (data not given).

6.3.2 Gene Expansion in Mycoparasitic Trichoderma *spp.*

Sequence similarities between orthologous and syntenic proteins in *T. reesei*, *T. atroviride*, and *T. virens* were 74 %, which resembles the evolutionary distance between Osteichthyes (bony fish) and Hominidae (Fedorova et al. 2008). The vast majority of the genes occur in all three *Trichoderma* species, but *T. atroviride* and *T. virens* share 1,273 orthologues that are not present in *T. reesei* and in addition contain 2,510 and 2,756 orphan genes, respectively. This is also reflected in the shared protein clusters (Fig. 6.1), which reveal 3,733 shared PFAM clusters (http://www.pfam.sanger.ac.uk), and only 124 and 116 unique for *T. atroviride* and *T. virens*, respectively, whereas *T. reesei* contains 775 unique clusters. The genes that were present in only *T. atroviride* and *T. virens* were enriched in PFAM protein domains for fungal specific Zn(2)Cys(6) transcription factors (PF00172, PF04082), solute transporters (PF07690, PF00083), and putative secondary metabolite synthesizing and modifying enzymes (oxidoreductases, monooxygenases, AMP activation of acids, phosphopantetheine attachment, and synthesis of isoquinoline alkaloids) (Kubicek et al. 2011).

Markov cluster algorithm (MCL) analysis of the three *Trichoderma* species, together with 44 related genomes that were available at November 4, 2012, in the JGI database (DOE JGI program MycoCosm http://www.genome.jgi.doe.gov/programs/fungi/index.jsf), shows that *T. virens* and *T. atroviride* have a unique genome inventory among all Pezizomycotina: they harbor the highest number of genes that encode proteins with ankyrin and HET/ankyrin/NACHT domains among all other fungi (Fig. 6.2). Ankyrin repeats play important roles in microbial pathogenesis in bacteria and have been suggested to also exert an important biological role in endosymbiosis of *Wolbachia* (Walker et al. 2007). Proteins containing the ankyrin domain have not been studied systematically in Pezizomycotina so far. A preliminary phylogenetic analysis of randomly chosen *Trichoderma* ankyrin proteins and 99 homologous fungal sequences encoding proteins with ankyrin domain revealed that the *Trichoderma* genes formed their own clades, suggesting that the ankyrin-domain proteins may evolve by extensive gene duplication. Since also the genomes of *T. harzianum* and *T. asperellum* harbor expanded sets of ankyrin-domain proteins (unpublished data), it is possible that these genes contribute to the unique opportunistic success of *Trichoderma*.

Genes encoding proteins with HET (heterokaryon incompatibility)/NACHT domains are part of the genetic systems that lead to recognition of and response to nonself during cell fusion between different individuals belonging to the same species. Fedorova et al. (2005) have proposed that the HET domain may represent a

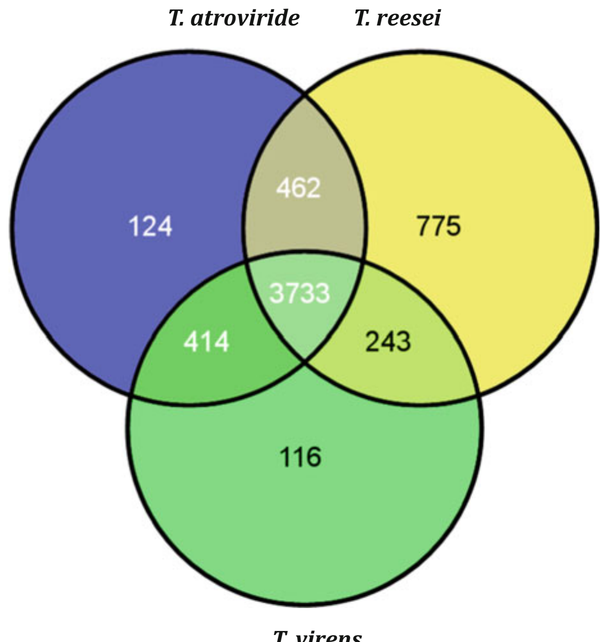

Fig. 6.1 Distribution of shared and unique orthologous Pfam families between *T*. *reesei*, *T*. *virens*, and *T*. *atroviride*. Ortho-MCL (http://www.orthomcl.cbil.upenn.edu) was used for identification of clusters in the three species

niche adaptation strategy of filamentous fungi to react to stimuli associated with defense against pathogens, self/nonself recognition, differentiation, or analogous roles. Paoletti and Saupe (2009) proposed that the *het* genes might also have a function in the recognition and response to pathogens. It will be interesting to investigate whether some of the HET proteins are involved in the reaction mechanisms during mycoparasitism in *Trichoderma*.

6.4 Specific Genomic Features

6.4.1 The Secretome of Trichoderma

Unlike most of higher organisms, fungi digest their food outside of their cells and to this end secrete enzymes and proteins for breaking down macromolecular nutrients and for the interaction with components of the environment that cannot penetrate through the cell wall. The inventory of secreted proteins may therefore be indicative of the habitat to which a given organism has become adapted. To this end, several studies used proteomic approaches to investigate *Trichoderma* spp. during saprotrophic growth, growth on lignocelluloses or chitin, and during interaction with other fungi and/or plants (Grinyer et al. 2005; Suarez et al. 2006; Marra et al. 2007). However, as we have previously discussed (Druzhinina et al. 2012),

function	**ankyrin_1**	**MFS_1**	**ADH_short**	**sugar transport**	**HET,** *PF05729, NACHT, PF00400, WD40*	**MFS_1**	**CFEM**	**cytochrome P450**	**MFS_1,** *PF00083, sugar transport*	**adh_short**	**HET**	**MFS_1**	**HET**	**aldehyde dehydrogenase**	**CO_esterase**	**ABC transporter,** *PF00664, ABC membrane domain*	**ADH_N-terminal,** *PF00107, ADH_zinc_N-terminal*	**TPR domain,** TPR_4,PF07720,TPR_3, PF06985, HET, PF07721	**sugar transport**	**RAS**	**PKS (see appendix)**	**NmrA** *isoflavon reductase (PF02716)*	**methyltransferase_12,** *PF08241, methyltransferase_11*
Tr	**32**	**34**	**40**	**39**	**15**	**31**	**29**	**25**	**17**	**19**	**11**	**25**	**13**	**20**	**13**	**13**	**15**	**2**	**11**	**21**	**9**	**10**	**18**
Tv	**118**	**57**	**54**	**51**	**51**	**47**	**46**	**44**	**36**	**34**	**33**	**31**	**30**	**30**	**28**	**25**	**24**	**24**	**23**	**22**	**22**	**22**	**21**
Ta	**99**	**52**	**52**	**46**	**41**	**53**	**32**	**28**	**39**	**29**	**29**	**30**	**34**	**24**	**20**	**13**	**25**	**16**	**18**	**21**	**14**	**24**	**18**
S. nod	17	75	48	64	0	41	76	54	28	13	30	25	50	19	21	9	10	4	16	20	16	8	13
S. popu.	1	33	49	32	0	31	22	31	9	5	1	9	3	15	12	5	9	0	6	20	6	2	9
F. oxy.	67	128	85	126	28	58	80	79	44	39	29	43	45	54	35	20	25	23	56	24	17	18	84
V. dal.	22	67	46	57	1	27	45	31	19	15	4	23	17	24	17	8	15	9	31	21	9	4	24
A. acu.	44	70	56	66	13	78	74	53	41	11	1	32	0	25	32	27	22	6	15	21	29	10	10
C. sat.	52	53	50	50	32	40	79	55	22	9	24	17	43	24	18	6	9	33	11	21	18	6	10
N. crassa	4	27	31	29	1	20	29	16	17	6	25	8	9	14	8	9	8	2	6	23	6	4	27
A. brass.	4	48	55	55	0	36	54	42	19	5	13	16	16	21	19	8	11	3	12	21	11	3	11
S. scler.	16	31	39	44	17	40	49	43	33	7	17	11	15	13	16	15	12	13	5	22	22	3	15
C. hetero.	45	53	57	51	16	44	77	60	24	14	19	20	36	27	17	7	12	20	13	21	21	7	9
M. grisea	18	55	42	44	1	23	86	52	32	13	13	13	18	17	17	12	7	4	14	22	29	7	16
Tuberm.	0	16	20	17	0	12	7	11	7	3	0	2	0	10	1	4	4	10	1	24	1	0	17
N. tetra.	6	26	27	30	2	21	23	16	17	6	23	8	8	13	8	9	8	3	6	20	6	4	28
C. zeae	11	59	73	51	1	44	42	45	21	8	6	16	21	26	14	6	14	0	9	21	8	6	15
N. haema.	42	161	100	121	18	56	94	79	51	42	129	42	99	66	34	17	32	12	65	24	12	28	90
L. macu.	3	43	36	50	2	30	48	27	15	4	10	15	14	17	18	5	7	1	6	22	14	4	8
A. nidulans	36	86	63	80	10	57	60	57	35	12	1	23	2	27	15	13	13	16	21	21	21	8	12
P. tritic	18	45	53	48	8	36	57	49	30	8	19	19	14	21	19	8	10	11	9	23	17	5	9
M. grami	11	57	65	45	3	43	23	40	17	9	2	18	21	20	16	14	11	2	13	22	10	5	12
B. cine.	28	39	50	58	9	51	54	59	50	16	32	27	27	14	36	17	14	10	6	21	26	5	14
C. globo.	33	28	32	36	15	26	47	30	29	7	10	16	17	12	15	11	7	20	7	18	25	4	21
A. carb.	24	63	72	71	6	88	84	63	37	20	6	36	12	33	29	23	20	7	13	22	21	12	13
C. heter.	53	55	57	51	22	44	82	62	27	14	23	20	38	27	18	7	12	22	13	22	20	7	10
A. niger	48	71	66	77	13	92	79	73	51	18	10	40	9	32	35	23	18	11	13	21	40	9	10
F. gram.	29	86	53	69	17	38	76	48	32	18	39	26	34	30	21	16	11	13	27	22	12	11	49
N. discreta	5	25	25	30	0	19	26	17	16	6	19	9	5	13	8	9	8	1	6	22	6	3	25
C. para.	33	48	44	58	2	51	92	54	55	23	39	19	47	19	29	15	13	5	13	25	29	9	16
PFAM	PF00023	PF07690	PF00106	PF00083	PF06985	PF07690	PF05730	PF00067	PF07690	PF00106	PF06985	PF07690	PF06985	PF00171	PF00135	PF00005	PF08240	PF00515	PF00083	PF00071	various	PF05368	PF08242

Fig. 6.2 Number of orthologous PFAM families in Pezizomycotina. Those that are significantly enriched in *Trichoderma* are given in *bold*

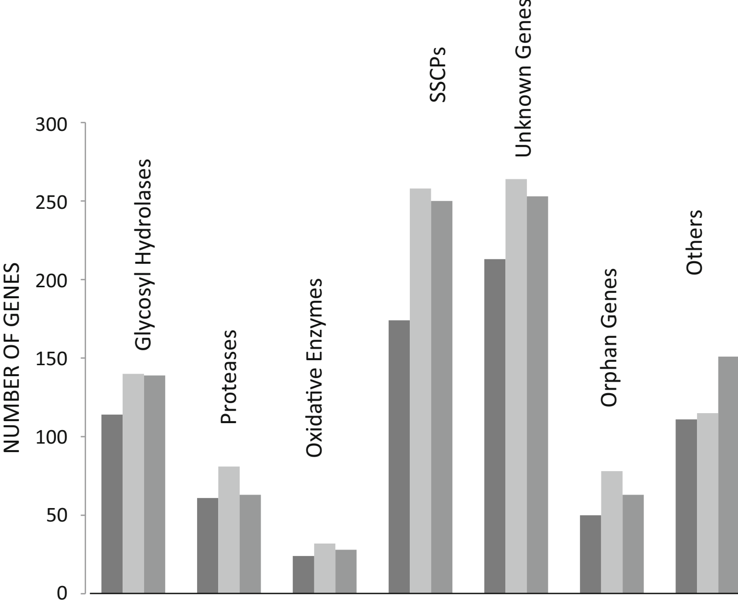

Fig. 6.3 Distribution of major gene families in the secretome from *T. reesei* (darkest bars), *T. atroviride* (lightest bars), and *T. virens*. Data are taken from Druzhinina et al. (2012)

these analyses provide only partial information. Here, we will concentrate only on the *in silico* identification of secreted proteins (Druzhinina et al. 2012): the version 2 annotations of the genomes of *T. reesei*, *T. atroviride*, and *T. virens* encode—after removal of membrane proteins—747, 968, and 947 proteins, respectively, that contain a statistically highly supported signal peptide and thus enter the secretory pathway. Roughly 85 % of them can be attributed to six groups (Fig. 6.3): carbohydrate-active enzymes (CAZys), proteases, small secreted cysteine-rich proteins (SSCPs), oxidative enzymes, hypothetical conserved proteins (i.e., proteins for which orthologues are found in other ascomycetes but whose amino acid sequence does not allow the prediction of a function), and orphan proteins (which are present only in *Trichoderma*). The high number of unknown proteins is striking, indicating that our knowledge on the proteins secreted by this fungus is very incomplete.

6.4.1.1 Small Secreted Cysteine-Rich Proteins (SSCPs)

As shown in Fig. 6.3, SSCPs comprise the largest group of proteins secreted by *Trichoderma*. They are identified by the criteria that (a) their M_r should be less than or equal to 300 amino acids long [as recommended by Martin et al. (2008)] and (b) containing four or more cysteine residues (Kubicek et al. 2011). Similarity searches and phylogenetic analyses allow their further subdivision into four groups:

(1) hydrophobins and hydrophobin-like proteins; (2) elicitor-like proteins; (3) proteins with similarity to MRSP1 (MAP kinase repressed secreted protein 1), a 16 kDa protein that was identified to be strongly overexpressed in a delta-tmk1 (a MAPK) mutant of *T. virens* and which bears the conserved four-cysteine pattern C-X29-C[P/G]C-X31-C; and (4) SSCPs for which no member with a known function has as yet been identified (Kubicek et al. 2011). Among them the hydrophobins, probably the best known SSCPs, are characterized by the presence of eight positionally conserved cysteine residues of which 4 occur in conserved C–C doublets. They are found on the outer surfaces of cell walls of hyphae and conidia, where they mediate interactions between the fungus and the environment, and are also secreted out of the cells to alter the hydrophobicity and/or hydrophily of solid structures in the habitat, for example at the attachment to plant roots (Viterbo et al. 2005) and to increase cutinase activities on hydrophobic polymeric substrates (Espino-Rammer et al. 2013). Hydrophobins differ in their solubility in solvents, hydropathy profiles, and spacing between the conserved cysteines, which led to the postulation of two classes, I and II. Among ascomycetes, *Trichoderma* is known to have the largest number of class II hydrophobins (Kubicek et al. 2008), and some of its subgroups were even shown to be under positive selection pressure (I.S. Druzhinina, unpublished data). In addition, *T. atroviride* and *T. virens*—but not *T. reesei*—have hydrophobins that not only fail to fit into the characteristics of class II but also deviate from the class I hydrophobins of other fungi in their hydropathy, cysteine spacing, and protein surface pattern. These predicted proteins thus form separate clades within ascomycete class I hydrophobins in phylogenetic analysis (Seidl et al. 2011). Because of the widespread applications of hydrophobins in biotechnology and biomedical technologies (Scholtmeijer et al. 2004), some of them (including HFB1 and HFB2 of *T. reesei*) are the subjects of structural and mechanistic investigations (Bayry et al. 2012).

A second SSCP group, for which interest has recently been emerging, is the elicitor-like proteins. They are frequently named "cerato-platanins" or "Snodprot" in the literature, due to their first description from *Ceratocystis fimbriata* and *Stagonospora nodorum* (Pazzagli et al. 2006). One of them, *T. virens* Sm1 and its *T. atroviride* orthologue EPL1, has been studied in some detail: EPL1 makes up for the major portion of proteins secreted by *T. atroviride* during growth on glucose (Seidl et al. 2008), and Sm1 induces systemic disease resistance in cotton and maize (Djonovic et al. 2006, 2007). It is therefore likely that the group 2 SSCPs are factors which aid in the interaction of *Trichoderma* with plants, as already shown for plant pathogenic fungi (Rep 2005). However, their exact role is difficult to predict because SSCPs are important for symbiotic interactions in the ectomycorrhizal basidiomycetes *Laccaria* (Plett et al. 2011). This high number of these SSCPs in *Trichoderma* and the functions already identified for these proteins in other fungi suggests that they could play a mutualistic role in *Trichoderma*, which may be in interaction with the plant, endophytism but also antagonization of fungi, bacteria, and higher soil organisms.

6.4.1.2 Polysaccharide Hydrolyzing Enzymes

Being non-plant pathogenic opportunistic saprotrophs and mycoparasites, *Trichoderma* spp. should be expected to possess a strong arsenal of extracellular enzymes capable of hydrolyzing both the polymers in their habitat (pre-decayed wood, soil litter) as well as for feeding on living or dead fungi. This expectation is nicely fulfilled in the case of the latter: the cell wall of asco- and basidiomycetes consists mainly of chitin and β-(1,3)-glucan (Latgé, 2007), and *Trichoderma*—and in particular *T. atroviride* and *T. virens*—consequently display a much higher number of glycoside hydrolase (GH) family 18 chitinolytic enzymes, GH75 chitosanases, and various β-1,3-glucanases families (GH17, GH55, GH64, and GH81) than other fungi. The properties of these hydrolases have been described in detail in several recent papers and reviews, and the reader is referred to them (Seidl 2008; Kubicek et al. 2011). Ihrmark et al. (2010) studied the evolutionary patterns of the chitinase genes *chi18-5*, *chi18-13*, *chi18-15*, and *chi18-17*, which all exhibit specific expression during mycoparasitism-related conditions, from 13 different *Trichoderma* spp. They found that two members (*chi18-15* and *chi18-17*) evolve under positive selection typical of rapid coevolutionary interactions, which underlines the importance of chitinases in mycotrophy and perhaps also in mycoparasitism.

The plant cell wall consists of the β-(1,4)-linked glucose polymer cellulose, hemicellulose polysaccharides of varying composition, and lignin. Like most ascomycetes, *Trichoderma* cannot degrade lignin. However, it secretes a powerful enzyme system for the degradation of cellulose and hemicelluloses which was the reason for developing *T. reesei* towards the industrial producer of these enzymes. It was therefore expected that *T. reesei* would also display a considerable amplification of the genes encoding the corresponding enzymes such as cellobiohydrolases and endo-β-1,4-glucanases. Somewhat disappointingly, however, the *T. reesei* genome contains only a minimal repertoire of these enzymes, two cellobiohydrolases (GH6 and GH7) and four endo-β-1,4-glucanases (GH5, GH7, GH12 and GH45) (Martinez et al. 2008), and these numbers are similar in *T. atroviride* and *T. virens* (Kubicek et al. 2011). This is in striking contrast to the cellulase repertoire of other saprophytic fungi-like *Aspergilli* and plant pathogenic fungi (Gibson et al. 2011). A very efficient system for induction of expression of these genes, rather than a large cellulase repertoire, can account for the high cellulolytic activity of *T. reesei*. A comparison with other fungi such as *Neurospora crassa* or *Aspergillus nidulans* reveals that in *T. reesei*, one single transcription factor (the Zn2Cys6-type transactivator XYR1) induces not only the expression of xylanase genes (as in *N. crassa* and *A. nidulans*) but also all cellulase genes and several more hemicellulase genes (Stricker et al. 2006; Akel et al. 2009; Bischof et al. 2013). The importance of this regulator is also reflected by the analysis of two cellulase hyperproducing mutants (RUT C30 and CL847), which were shown to display a deregulated and increased expression of *xyr1*. This coregulation of cellulases and hemicellulases may be further augmented by another feature that distinguishes the

genomes of *T. reesei* (and also *T. atroviride* and *T. virens*; unpublished data) from that of other Pezizomycotina: the occurrence of the cellulase and hemicellulase genes in clusters (Martinez et al. 2008). In *T. reesei*, 130 of the 316 (41 %) of the GH and other carbohydrate-active proteins (such as glycosyl transferases, carbohydrate esterases, and carbohydrate-binding proteins; further termed "CAZyme" genes) occur in 25 discrete regions ranging from 14 kb to 275 kb in length at an average density of fivefold greater than the expected density for randomly distributed genes. Most of these clusters occur near the end of the respective *T. reesei* scaffolds and within or near nonsyntenic genomic areas (Kubicek 2012). In particular, the genes encoding cellulose-binding proteins are exclusively found in the syntenic gaps (Martinez et al. 2008). This suggests that these CAZymes are either located near the chromosomal ends or in dynamic (repeat-rich) regions of the genome.

The degradation of lignocelluloses by cellulases and hemicellulases could be further augmented by oxidative enzymes: the *Trichoderma* secretome contains a number of oxidative enzymes, including glucose oxidases, multicopper oxidases (including laccases), and copper radical oxidases (glycolate oxidases), whose induction by cellulose is accompanied by a strong upregulation of genes encoding iron-uptake systems, ferric reductases, and oxalate decarboxylases (Bischof et al. 2013). Together, these proteins could perform an oxidative attack on the cellulose, thereby increasing the number of amorphous areas that serve as places for attack by cellulases. This could also balance the low number of polysaccharide monooxygenases (formerly called GH61 endo-β-1,4-glucanases), of which *Trichoderma* possesses only a comparatively low number.

Despite the small number of cellulases in *T. reesei*, all three *Trichoderma* spp. are enriched in some hemicellulolytic components, such as GH27 α-galactosidases, GH30 glucuronyl-xylanases, GH43 α-arabinofuranosidases/β-xylosidases, GH67 and GH79 α-methyl-glucuronidases, and GH95 α-fucosidases (Kubicek 2012). This suggests that *Trichoderma* has apparently specialized towards efficient hydrolysis of some hemicelluloses side chains. Interestingly, several of these enzymes are encoded by genes that share no homologues in other fungi but only in bacteria. These genes may thus have been obtained by horizontal gene transfer, further stressing that they are important for successful competition in the natural habitat of *Trichoderma*.

The reduced set of enzymes involved in the degradation of pectin (GH28 polygalacturonases and pectin/pectate lyases) is of further interest: a functional analysis of an endopolygalacturonase gene from *T. cf. harzianum* T34 showed that the enzyme is required for root colonization but does not induce plant defense reactions (Moran-Diez et al. 2009). The reduced activity of *Trichoderma* on pectin may thus minimize plant defense reactions and thereby facilitate the interaction of *Trichoderma* with plants.

6.4.1.3 Proteolytic Enzymes

Trichoderma also secretes about 20 % of its proteases into the medium (Druzhinina et al. 2012). Their composition is dominated by aspartyl proteases, methionyl peptidases, aminopeptidases S, subtilisin peptidases, dipeptidyl peptidases, sedolisins, and prolyl peptidases. Some of these genes are highly expressed during antagonism of *Rhizoctonia solani* by *T. atroviride* and *T. virens* (Seidl et al. 2009a, b; Atanasova et al. 2012), and knockout strains in some proteases showed a reduced antagonistic reaction (Benitez et al. 2004). There is also some evidence that proteases may be involved in the attack of nematodes, and it was speculated that they may aid in the interaction with human tissues (Kredics et al. 2004). Proteases are also an important issue in the enzyme producer *T. reesei*, where they can lead to massive degradation of the proteins secreted by the fungus. The regulation of protease production by *Trichoderma* has not yet been investigated but obviously presents a major gap in our understanding of this fungal genus.

6.4.2 Trichoderma *Genes for Secondary Metabolites*

Numerous secondary metabolites from various *Trichoderma* spp. have been reported [for review, see Shivasithamparam and Ghisalberti (1998)], but due to a high level of uncertainty about the true species nature of the producing organism (most strains were identified only according to superficial morphological characters), their production by *T. reesei*, *T.virens*, and *T. atroviride* cannot be deduced. Also the genes for synthesis of these metabolites have in most cases not been identified. Therefore, *in silico* identification of secondary metabolite genes from genome data can only be performed for gene families that comprise typical and unique features: this criterion is fulfilled with the nonribosomal peptide synthetases (NRPS) and the polyketide synthases. *T. reesei*, *T. virens*, and *T. atroviride* contain 11, 18, and 18 PKS gene, respectively. While the numbers for PKS are average within fungi, the NRPS genes of *Trichoderma*—particularly of *T. virens*—are among the highest of all fungi. We have recently shown that this is apparently due to recent duplications of cyclodipeptide synthases, cyclosporin/enniatin synthase-like proteins, and NRPS hybrid proteins (Kubicek et al. 2011).

6.4.2.1 Nonribosomal Peptide Synthases

The genomes of *T. reesei*, *T. atroviride*, and *T. virens* harbor 10, 16, and 28 NRPS-encoding genes, respectively (Kubicek et al. 2011). Half of the NRPS genes present in *T. atroviride* or *T. virens* are unique for the respective species and occur within nonsyntenic islands of the genome, indicating their origin by recent genome rearrangements, which is also reflected in a higher nucleotide dissimilarity (about

30 %) than the average of genes between *T. atroviride* and *T. virens*. A unique feature of *Trichoderma* and close relative fungal families (Hypocreaceae, Clavicipitaceae, and Bionectriaceae) is the presence of two NRPSs that synthesize modified peptides, termed peptaibols: these are small (500–2,000 Da) linear peptides containing a high number of non-proteinogenic, α,α'-dialkylated α-amino acids like isovaline and α-aminoisobutyric acid (Aib). In addition, their N-terminal amino acid is acetylated and the C-terminus is reduced to an amino alcohol, mostly phenylalaninol. These properties have given rise to the name peptaibol (*pept*ide, *Aib*, and amino alcoh*ol*). Peptaibols form a helical structure with the hydrophobic side chains exposed to the surface. This structure allows them to interact with natural and artificial bilayers to form pores or voltage-dependent ion channels, increasing membrane permeability (Rebuffat et al. 1999). The structure and properties of more than 700 peptaibols are collected in the Peptaibol Database (http://www.peptaibol.cryst.bbk.ac.uk/home.shtml), and details of their biochemistry and structure are reviewed by Duclohier (2007).

Of the two genes encoding peptaibol synthases in *Trichoderma*, one synthesizes the short (10–14 aa) and one the long (18–25 aa) peptaibols. Interestingly, the former synthase synthesizes two small peptaibols (11 and 14 kDa; Mukherjee et al. 2011; Degenkolb et al. 2012) which has been attributed to module skipping (Mukherjee et al. 2011; Degenkolb et al. 2012) and could be further kinetically regulated by the chemical structure of the intermediate peptides. By this mechanism, the two synthases give rise to the multitude of peptides produced by these enzymes depending on the available precursor concentrations. As an example, Stoppacher et al. (2008), using LC-MS, detected 20 trichorzianes and 15 trichoatrokontins (representing the high and smaller peptaibols, respectively) in culture filtrates of *T. atroviride*.

Two other NRPSs, for which genes are present in *T. virens* and *T. reesei*, but not in *T. atroviride*, synthesize the epipolythiodioxopiperazine-type peptides gliotoxin and gliovirin (Patron et al. 2007). The former is exclusively produced by so-called "Q-strains" of *T. virens*, whereas the latter is only produced by "P-strains" of *T. virens* (Mukherjee et al. 2012a, b). Gliotoxin has fungistatic action and is also known from the opportunistic human pathogen *Aspergillus fumigatus* (Eurotiales, Ascomycota) where it has been discussed as a virulence factor (Dagenais and Keller, 2009). Its toxicity is due to the presence of a disulfide bridge in the molecule which can inactivate proteins via reaction with thiol groups and generate reactive oxygen species by redox cycling (Gardiner et al. 2005). Gliovirin has antimicrobial properties particularly against Oomycota (Mukherjee et al. 2012a).

The *T. virens* strain whose genome has been sequenced is a "Q-strain" (*T. virens* Gv29-8), and genomic data are therefore only available for gliotoxin biosynthesis. The synthase and the auxiliary biosynthetic enzymes are located in a cluster containing eight genes that are closely similar to those of *A. fumigatus* (Patron et al. 2007). Transcriptomic data during the confrontation of *T. virens* with *R. solani* have shown that gliotoxin formation is an early and major trait in the antagonism (Atanasova et al. 2012): almost all genes required for its biosynthesis were upregulated. These include genes involved in the production of the precursor of

gliotoxin, L-phenylalanine, synthesis of the glutathione required for the formation of the central disulfide bond, and *gliT*, encoding a thioredoxin reductase that has been shown to protect *A. fumigatus* against its own gliotoxin (Schrettl et al. 2010). These data nicely complement earlier findings of detection of gliotoxin in the rhizosphere (Lumsden et al. 1992), and it may thus be the major principle in the antagonistic interaction of *T. virens* "Q-strains" with their preys.

Two NRPS of *Trichoderma* encode siderophores. Lehner et al. (2013) reported that *Trichoderma* spp., despite having only two genes, produced 12–14 siderophores, with six common to all species tested. The highest number (15) of siderophores was detected for *Trichoderma harzianum*, whereas *T. reesei* had the most distinctive pattern, producing one unique siderophore (cis-fusarinine) and three others that were present only in *T. harzianum* and not in other species. These data suggest that the high diversity of siderophores produced by *Trichoderma* spp. is—like in the case of the peptaibols—the result of further modifications of the nonribosomal peptide synthetase (NRPS) products.

None of the other *Trichoderma* NRPSs have as yet been characterized and consequently their products are unknown.

6.4.2.2 Polyketide Synthases

Out of a total of 47 PKS-encoding genes in the three sequenced *Trichoderma* spp., 29 fall into a single orthologous group (Baker et al. 2012). The loci of these PKSs are frequently flanked by cytochrome P450 monooxygenases, FAD-dependent monooxygenases, short-chain dehydrogenases/reductases, or epimerases next to PKS-encoding genes, suggesting that they are auxiliary components in the biosynthesis of the respective metabolites. Three PKS genes are induced during interaction of *T. atroviride* with *R. solani* and may therefore be involved in its antagonistic activities (Atanasova et al. 2013a, b). The *T. reesei* PKS gene Trire2:82208 has been shown by gene deletion to be responsible for the green color of the conidia but interestingly also to be involved in protection against other fungi but less in the direct attack on them (Atanasova et al. 2013a, b).

The genomes of the three *Trichoderma* spp. also harbor genes encoding mixed PKS-NRPS hybrids (2, 4, and 4 in *T. reesei*, *T. atroviride*, and *T. virens*, respectively). A mutation in one of the PKS-NRPS hybrid genes impaired the ability of *T. virens* to induce the defense response gene encoding phenylalanine ammonia lyase in maize roots, suggesting a putative role for the associated metabolite product in ISR (Mukherjee et al. 2012b).

6.4.2.3 Isoprenoid Derivatives

In addition to the products of NRPS and PKS, microorganisms and plants also synthesize secondary metabolites from isoprenoid precursors. The genomes of *T. reesei* and *T. atroviride* do not contain genes that can obviously be related to

these activities. However, Mukherjee et al. (2006) identified a cluster in the genome of *T. virens* that included three cytochrome P450 genes and one terpene cyclase. Because these genes are underexpressed in the mutant that is unable to produce viridin and viridiol, they proposed that this cluster could be responsible for the production of viridin.

One of the more prominent isoprenoid-derived secondary metabolites claimed to be synthesized by *Trichoderma* are trichothecenes. These are compounds that comprise a family of over 200 secondary metabolites with a common tricyclic 12,13-epoxytrichothec-9-ene (EPT) core structure and which are reported to be produced by species in at least six genera of the fungal order Hypocreales (class Sordariomycetes): *Fusarium*, *Myrothecium*, *Spicellum*, *Stachybotrys*, *Trichoderma*, and *Trichothecium*. The structure of trichothecenes identifies them as small, amphipathic molecules that can pass cell membranes by passive diffusion, which are therefore easily absorbed via the skin and gastrointestinal systems and result in a rapid effect on fast growing cells and tissues (Wannemacher and Winer 1977; McCormick et al. 2011). Exposure to trichothecenes can cause a number of symptoms, from feed refusal, immunological problems, vomiting, skin dermatitis, to immunosuppressive effects and neurotoxicity (Ueno 1985).

The precursors of trichothecene biosynthesis are formed in the isoprenoid biosynthetic pathway, from which the specific trichothecene biosynthetic pathway branches off at farnesyl pyrophosphate by the action of the enzyme trichodiene synthase (termed “Tri5” in *Fusarium* spp.) which catalyzes the cyclization of farnesyl pyrophosphate to trichodiene (McCormick et al. 2011). Trichodiene then undergoes a series of oxygenations catalyzed by the cytochrome P450 monooxygenase TRI4 to finally form the intermediate isotrichotriol. Detailed reviews on the biosynthesis of trichothecenes have been published, and the reader is referred to these sources for details [for review, see McCormick et al. (2011)].

The reputation of *Trichoderma* as a trichothecene producer is due to the problems with *Trichoderma* species identification (see Sect. 6.2.1): trichodermin was isolated from “*Trichoderma viride*” (Godtfredsen and Vangedal 1965); Watts et al. (1988) reported the production of trichodermin by “*T. reesei*,” and Corley et al. (1994) isolated the “harzianum A” from *T. harzianum*. The above named “*T. viride*” isolate is not available, and its identity thus cannot be reassessed. As for *T. reesei*, its genome sequence lacks a *tri5* orthologue, so that, in principle, it is unable to initiate trichothecene biosynthesis, a fact that is also underscored by the absence of trichothecene metabolites in culture filtrates of *T. reesei* QM 9414 grown on various different conditions (M. Sulyok and C.P. Kubicek, unpublished data). As for the harzianum A producer “*T. harzianum*,” Nielsen et al. (2005) proved that this strain is in fact *T. brevicompactum*. They investigated several molecularly assessed *Trichoderma* spp. and found that species from the *T. brevicompactum* clade (*T. brevicompactum*, *T. arundinaceum*, *T. turrialbense*, and *T. protrudens*) are the only source of trichothecene production within the genus *Trichoderma*.

6.4.2.4 Volatile Secondary Metabolites

In addition to terpenoids, several other volatile metabolites have been reported to be produced by *Trichoderma*. A recent thorough investigation has been done on *T. atroviride* (Stoppacher et al. 2010). Unfortunately, there is hardly any information available on the genes of their synthesis. 6-n-pentyl-2H-pyran-2-one (6PP), a volatile component with coconut aroma and antifungal activity, is one of the most well-studied secondary metabolites from a biocontrol perspective (Bonnarme et al. 1997; Cooney et al. 2001; Reithner et al. 2005, 2007; Vinale et al. 2008). 6PP formation has been detected in *T. atroviride*, but not in *T. reesei* and *T. virens* (Reino et al. 2008). Its biosynthesis may occur via linoleic acid, analogous to jasmonate synthesis in which the necessary hydroxyl group originates from an oxidation by lipoxygenase (Serrano-Carreon et al. 1993). Atanasova et al. (2012) detected a lipoxygenase gene (Triat1:33350) that is present in *T. atroviride* and not in *T. reesei* and *T. virens*. It is overexpressed during contact with *R. solani* (Atanasova et al. 2012) in correlation with the characteristic coconut smell of the *T. atroviride* cultures (Aghcheh et al. 2013).

6.5 Genome Sequencing of *T. reesei* Cellulase-Producing Mutants

The availability of the *T. reesei* wild-type (QM 6a) genome sequence and the progress in high-throughput massive parallel sequencing technologies made it possible to also sequence the genomes of several mutant strains that are either improved or defective in cellulase production (Fig. 6.4). Le Crom et al. (2009) compared the genome sequence of *T. reesei* QM 6a (Martinez et al. 2008) with two isolates of the improved producer lineage developed at Rutgers University, NG14, and RUT C30 (Eveleigh and Montenecourt 1979). Three mutations (a truncation of the *cre1* gene encoding the carbon catabolite repressor CRE1, Ilmen et al. (1996); a frame shift mutation in the glucosidase II alpha subunit gene *gls2* involved in protein glycosylation which increases protein secretion, Geysens et al. (2005); and an 85-kb deletion that eliminated 29 genes, including transporters, transcription factors, and primary metabolic enzymes, Seidl et al. (2008)) had previously been described to occur in strain RUT C30. The comparison of the genomic sequence of strains NG14 and RUT C30 with that of QM 6a identified a further 18 and additional 25, respectively, non-synonymous mutations in them. Nine of the 18 mutated genes in *T. reesei* NG14 are involved either in RNA metabolism (3 genes), protein secretion/vacuolar targeting (3 genes), or transcriptional regulation (3 genes). Genes affected in RUT C30 partially fall into the same categories but also include genes involved in sugar transport and general metabolism (8 genes), probably due to the selection for growth on glycerol in the presence of 2-deoxyglucose (Eveleigh and Montenecourt, 1979). Vitikainen et al. (2010),

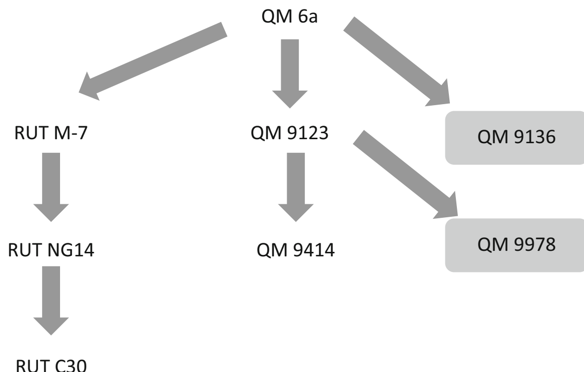

Fig. 6.4 Genealogy of *T. reesei* mutants, whose genome has already been sequenced. Strains over *gray* background specify cellulase-negative strains

using tiling arrays and comparative genomic hybridization, identified additional 12 and 4 mutations in NG14 and RUT C30, respectively. These mutations, besides unknown genes, comprised a transcription factor and a clathrin complex subunit in NG14 and another transcription factor and a Gβ-WD40 protein in RUT C30. A systematic functional analysis of these genes is currently under investigation in our laboratory and may potentially identify novel components relevant to cellulase production.

A reverse approach is the sequencing of mutants that are defective in cellulase production. The genomes of two such strains have also been sequenced: *T. reesei* QM 9136, which is unable to grow on cellulose and does not secrete any cellulases when grown on soluble cellulase inducers (Mandels et al. 1971), and *T. reesei* QM 9978, which is also unable to grow on cellulose, but which grows and secretes cellulases on soluble cellulase inducers (Zeilinger et al. 2000). In the case of QM 9136, the key mutation was identified as a frame shift in the cellulase regulator gene *xyr1* which leads to a C-terminally truncated protein and whose cellulase-negative phenotype can be recovered by transformation with the native *xyr1* gene. The mutations present in QM 9978 are less straightforward to interpret and are the subject of current investigations.

6.6 Benefits From the *Trichoderma* Genome Sequences

Obviously, the availability of genome sequences provides only an overview about the genetic repertoire of an organism, and an interpretation of the differences from other organisms requires the integrated use of other "-*omic*" techniques (i.e., systems biology) and availability of high-throughput techniques to manipulate and investigate genes. Due to the strong industrial interest, these have so far most advanced in *T. reesei*: oligonucleotide arrays and tiling arrays have been constructed for genome-wide analyses of gene expression under different cellulase and hemicellulase-inducing conditions (Häkkinen et al. 2012; Ivanova et al. 2013), under conditions of asexual sporulation (Metz et al. 2011) and UV-light signaling

(Tisch et al. 2012). Microarray strategies were also devised to understand the consequences of mutations in regulator genes (such as those encoding the carbon catabolite repressor CRE1 or the protein methyltransferase LAE1, which is believed to regulate the transcriptional activity of chromatin; Portnoy et al. 2011a, b; Seiboth et al. 2012; Karimi-Aghcheh et al. 2012). The genome sequence was also used for ChIP-seq with antibodies against histone modifications known to be associated with transcriptionally active (H3K4me2 and -me3) or silent (H3K9me3) chromatin, in order to study chromatin changes during cellulase production (Aghcheh et al. 2013). In combination with proteomic and metabolomic investigations, these data can be used for relating protein production rate to metabolic activities (Arvas et al. 2011). Also, manipulation of genes that are significantly upregulated under these conditions led to the identification of new regulators of cellulase and hemicellulase production (Ivanova et al. 2013).

To support such investigations, systems for high-frequency homologous transformation have been developed: fungi contain two pathways for integration of uptaken DNA, one leading to homologous and one leading to heterologous integration. Guangtao et al. (2009) and Steiger et al. (2011) deleted one of the genes involved in ectopic integration (the orthologues of yeast *ku70* or human *LIG4*). The corresponding strains display more than 90 % integration of transformed DNA into the homologous locus. By employing a Cre/loxP-based excision system, both marker insertion and marker excision can be positively selected for by combining resistance to hygromycin B and loss of sensitivity to fluoroacetamide, thus enabling an unlimited use of the same markers. Alternatively, a blaster cassette has been developed which also enables unlimited marker recycling (Hartl and Seiboth 2005). Schmoll et al. (2012) have integrated the targeted transformation system into a high-throughput gene knockout system which is based on yeast recombination for fast vector synthesis and a genome-wide database of primers for amplification of the respective gene fragments.

Corresponding developments with *T. virens* and *T. atroviride* lag somewhat behind *T. reesei*. Towards studying the interaction of *Trichoderma* with plants, E. Monte and coworkers designed arrays consisting of a combination of *T. reesei* gene oligonucleotides and probes based on ESTs from seven different *Trichoderma* spp. (including *T. cf. harzianum* CECT2413, *T. atroviride*, and *T. asperellum* grown in the presence of plant pathogen cell walls). Samolski et al. (2009) investigated gene expression of *T. cf. harzianum* CECT2413 when contacting the roots of tomato plants using these arrays. They detected transcripts putatively encoding proteins related to *Trichoderma*-host (fungus or plant) associations, such as the SSCPs Sm1/Elp1 and QID74, proteases, endochitinases, biosynthesis of nitric oxide, xenobiotic detoxification, mycelium development, and those responsible for formation of infection structures in plant tissues. Rubio et al. (2012)—working with *T. harzianum* CECT 2413 (T34), *T. virens* Gv29-8 (T87), and *T. hamatum* IMI 224801 and using the same arrays—showed that after 20 hours of incubation in the presence of tomato plants, genes involved in chitin degradation (i.e., *N*-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, and some chitinases) were the most significantly upregulated.

Towards the identification of genes related to mycoparasitism in *T. atroviride*, Reithner et al. (2011) used a high-throughput sequencing approach to analyze the changes in the transcriptome of *T. atroviride* IMI 206040 during interactions with *R. solani*. The most abundant KOG group found during direct contact was "metabolism." Relative gene expression analysis of these genes, conducted at different stages of mycoparasitism against *Botrytis cinerea* and *Phytophthora capsici*, revealed a synergistic transcription of various genes involved in cell wall degradation. Atanasova et al. (2012) compared the transcriptional responses of *T. atroviride* IMI 206040 and *T. virens* Gv29-8 with that of *T. reesei* QM 6a during confrontations with a plant pathogenic fungus *R. solani*. They found that the three *Trichoderma* spp. exhibited a strikingly different transcriptomic response already before physical contact with alien hyphae. *T. atroviride* expressed an array of genes involved in production of secondary metabolites, GH16 β-glucanases, various proteases, and SSCPs. *T. virens*, on the other hand, expressed mainly the genes for biosynthesis of gliotoxin, respective precursors, and also glutathione, which is necessary for gliotoxin biosynthesis. In contrast, *T. reesei* increased the expression of genes encoding cellulases and hemicellulases and of the genes involved in solute transport. Thus, the initial *Trichoderma* mycotrophy has differentiated into several alternative ecological strategies ranging from parasitism to predation and efficient saprotrophy.

Clearly, these studies only provide initial snapshots of the transcriptional processes that go on during the interaction of *Trichoderma* spp. with plants or other fungi, and functional gene analysis will be necessary to reveal the mechanisms that are involved. To this end, Catalano et al. (2010) constructed a *ku70*-deficient strain of *T. virens* which can be used for high-throughput gene deletion analysis. A first approach towards a more comprehensive use of *T. atroviride* transcriptomics has been recently published by Hernández-Oñate et al. (2012), using high-throughput RNA-seq analyses of the triggering of conidiation by mechanical injury. Gene-replacement experiments demonstrated that injury triggers NADPH oxidase (Nox)-dependent ROS production and that Nox1 and NoxR are essential for asexual development in response to damage.

6.7 Concluding Remarks

Although *Trichoderma* does not have the glorious biological history of *Neurospora* or *Aspergillus*, and its research community is consequently smaller, it is nevertheless of high importance to mankind in several ways: *T. reesei* is one of the leading filamentous fungi for enzyme and heterologous protein producing biotechnology, and other species are employed as versatile fungicides and phytostimulating agents. It is therefore not surprising that the three species that have been sequenced and annotated (*T. reesei*, *T. virens*, *T. atroviride*) and two of those whose genome sequences have recently been released (*T. asperellum*, *T. harzianum* sensu stricto) are relevant to these two areas. While their analysis is still only at the beginning, the

sequence of further species such as *T. longibrachiatum* and *T. citrinoviride* will help to answer the question whether the ability to interact with animals is due to the gain or loss of genes or a change in their regulation and—a significant difference from the other species sequenced so far—what makes them capable of growing at the body temperature of mammals. In addition, the further sequencing of *Trichoderm*a species that are obligate endophytes [e.g., see Druzhinina et al. (2011)] may contribute to a deeper understanding of the mechanisms involved in *Trichoderma*-plant interaction.

As for future research perspectives, the already available data offer a room for basic and applied science. As an example, the large number of genes encoding oxidative enzymes (cytochrome P450 monooxygenases, FAD-linked oxidases/ monooxygenases), methyltransferases, esterases, and transcription factors and which are often found in clusters in the genome may encode enzymes for the synthesis of unknown secondary metabolites. Aghcheh et al. (2013) have shown that overexpression of the *lae1* gene activates the expression of some silent secondary metabolite genes in *T. reesei*, and this tool may aid in the above attempts.

Acknowledgements Genome sequencing and analysis were supported by the Office of Science of the US Department of Energy under contract number DE-AC02-05CH11231. The authors own work on this topic was supported by grants from the Austrian Science Fund P-17895 to I.S.D.

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Chapter 7
Fusarium oxysporum: A "Moving" View of Pathogenicity

Apratim Chakrabarti

7.1 Introduction

The genus *Fusarium* is a large, complex one, and the saying goes that if something is green, then there is some *Fusarium* growing on it, in it and with it. The diversity of hosts that *Fusarium* attacks, the number of pathogenic taxa and the range of habitats in which they cause disease are the greatest in plant pathology. This highly diverse genus contains some of the most notorious plant pathogens that affect agricultural and horticultural crop productions globally. Examples of havoc caused by this disease include near wipeout of the banana industry by *Fusarium* (Panama) wilt in the 1960s (Stover and Ploetz 1990) to recent outbreaks of *Fusarium* head scab in wheat (Kazan et al. 2012). Members of this genus are important human pathogens and is the second most important mould causing infections in immunocompromised patients, often with a lethal outcome (Ortoneda et al. 2004).

More than three centuries after the introduction of this genus, the great diversity present within *Fusaria* represents a formidable challenge for classifying its members into distinct species. Delineation depended on morphological features, biological species concepts and, more recently, phylogenetic studies involving polymorphisms in several gene sequences (Summerell et al. 2010; Taylor et al. 2000). Depending on the methods applied, the number of identified species ranged from 9 to over 1,000 (Leslie et al. 2006). At the time of writing this review, the Fusarium-ID database (http://isolate.fusariumdb.org/index.php) lists 76 distinct

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_7,
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species of *Fusarium* based on sequence of the translation elongation factor 1 alpha (EF-1α) gene (Geiser et al. 2004).

The most significant development in *Fusarium* research in recent times has been the availability of whole genome sequences. In 2007, the *F. graminearum* genome sequence was first published (Cuomo et al. 2007b), and the Broad Institute released a comparative genomics database containing assembled and annotated whole genome sequences of *F. graminearum* (resequenced), *F. verticillioides* and *F. oxysporum* (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html). Soon after, the *F. solani* (teleomorph *Nectria haematococca*) whole genome sequence became available (Coleman et al. 2009; http://genome.jgi-psf.org/Necha2/Necha2.home.html). Availability of these high-quality, assembled and annotated genome sequences has greatly facilitated both forward and reverse genetic approaches to identify pathogenicity genes, novel modes of plant–pathogen interaction as well as evolution of *Fusarium* species as a group.

This chapter will focus on the species complex *Fusarium oxysporum*, the most commonly studied group causing vascular wilt diseases in a large number of plants. *Fusarium oxysporum* is well represented amongst soilborne fungi and is present in all types of soil across the globe. While all the strains exist saprophytically, some are well known for their capabilities to infect and induce wilt in plants, while others are not pathogenic to crops from whose asymptomatic roots (or rhizospheres) they were isolated (Gordon and Martyn 1997). The disease-causing isolates have been of natural interest to plant pathologists, and more than 100 different crops have been identified as hosts to *F. oxysporum* (Dean et al. 2012). In contrast to this broad host range at the species level, host specificity exists at the forma specialis level. Designation of forma specialis is primarily a description of host-infection function of an isolate rather than a taxonomical one and geneological discordance and conflicting relation between different formae speciales have been observed when sequence data from translational elongation factor 1-α and ribosomal intergenic spacer region were analysed (O'Donnell et al. 2009). Isolates within a given forma specialis group infecting one particular plant species can be of single or multiple clonal lineage (mono- and polyphyletic) (O'Donnell et al. 1998; Skovgaard et al. 2001). With the help of molecular markers, it was shown that isolates from different formae speciales can be more closely related than isolates within a forma specialis (Fourie et al. 2009, 2011). This polyphyletic origin of isolates within a forma specialis suggests that pathogenicity has independently evolved several times. Given that *F. oxysporum* predominantly, if not exclusively, reproduces through asexual means, the origin of pathogenicity in *F. oxysporum* is highly intriguing.

At the sub-forma specialis level, isolates are classified into races based on cultivar specificity of the host genotype. This corresponds with the gene-for-gene hypothesis for host resistance and has been well documented in the case of tomato and *F. oxysporum* f. sp. *lycopersici* (Takken and Rep 2010). The other classification widely used at the sub-forma specialis level is vegetative compatibility group (VCG). Isolates that can form a stable heterokaryon when two hyphae are fused are grouped under the same VCG, while isolates that cannot do so are under different VCGs. VCG grouping can identify isolates that share common alleles at

the VCG determining loci. This does not necessarily imply that they share the same lineage (Leslie et al. 2006).

7.2 Plant Infection by *F. oxysporum*

F. oxysporum can survive in the soil for extended periods of time as chlamydospores and is notoriously difficult to eliminate once soil is infested (Agrios 2004). Despite the large number of plants infected by *F. oxysporum*, disease incidence and mechanisms of plant infection have been studied in a limited number of plant species, e.g. tomato, cotton, banana, melon, bean, chickpea and more recently *Arabidopsis thaliana*. The process of plant infection has been visualised using light, fluorescence, electron and multiphoton microscopy(Bishop and Cooper 1983, 1984; Czymmek et al. 2007; Lagopodi et al. 2002; Olivain and Alabouvette 1999; Olivain et al. 2006). Dormant *F. oxysporum* chlamydospores present in the soil germinate under favourable conditions and infect plants through their roots. Initially the hypha attaches itself on the root surface and grows along the junctions of epidermal cells to form a dense network intermingled with root hairs. Following surface colonisation, direct penetration of the epidermal cells takes place. Although penetration occurs primarily at root tips, penetrations at random positions along root hairs as well as in the meristematic zones of primary and lateral roots have all been reported. However, no specialised penetration structure has so far been observed. Following penetration, the fungus continues to grow both inter- and intracellularly through the cortex tissue until it reaches xylem vessels. Entry into xylem vessels occurs through pits, and the fungus switches to a distinctive vascular phase where it remains confined within the xylem vessels. At this stage, the fungus proliferates within the xylem vessel and pervades through production of microconidia that spread upwards with xylem sap flow (Fig. 7.1). In the pre-vascular and vascular stages, plants respond to fungal invasion through activation of defence response mechanisms which include production of callose deposition, accumulation of phenolics, formation of tyloses (outgrowths of adjoining xylem parenchyma cells through pits) and gels in infected cells (Beckman 2000; Beckman and Roberts 1995). Characteristic wilt symptoms appear as a result of severe water stress caused by fungal proliferation combined with host-defence responses. At later stages of infection, as the plant dies, the fungus switches from a biotrophic to a necrotrophic mode, invades the host parenchyma and sporulates profusely. The whole process is highly coordinated and involves recognition of the host root, penetration, suppression and/or evasion of host-defence responses, toxin production, sporulation and growth. Cell-wall-degrading enzymes, enzymatic detoxification of phytoalexins, signalling pathways through mitogen-activated protein kinases (MAPK) and G-proteins, peroxisome function, multiple transcription factors and more recently effector

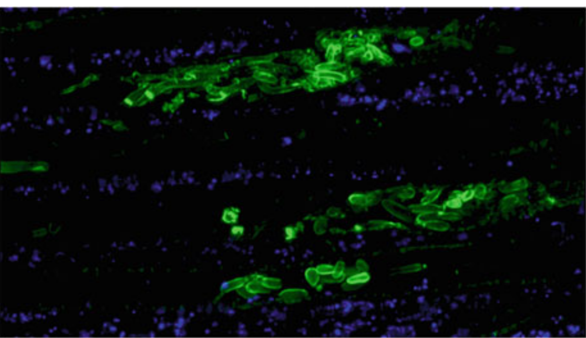

Fig. 7.1 *F. oxysporum* f. sp. *vasinfectum* growing inside xylem cells of infected cotton stem (longitudinal section). The fungus was stained with FITC-conjugated wheat germ agglutinin and observed using epifluorescence microscopy

proteins have all been implicated in the process of plant infection (Di Pietro et al. 2003; Michielse and Rep 2009).

7.3 Sensing the Environment

The first and foremost critical step in plant infection is sensing stimuli from plants and responding with appropriate physiological and morphological changes. These responses include spore germination, directed hyphal growth, adhesion to plants, development of specialised infection structures and expression of lytic enzymes and involve or are accompanied by changes in gene expression patterns triggered through signalling pathways.

One environmental condition that *F. oxysporum*, like all other microorganisms, must adapt to for its growth and proliferation is external pH. It responds to external pH by tailoring expression of genes encoding proteins that are directly exposed to the environment such as permeases, secreted enzymes and intercellular enzymes involved in production of secreted toxins, antibiotics or compounds that can influence external pH. A key component for adaptation to extracellular pH is PacC, a Cys2-His2 zinc finger-type transcription factor (Peñalva et al. 2008). PacC has been studied in great detail in *Aspergillus nidulans* where it controls expression of number of genes including phosphatases, xylanase, GABA permease and genes involved in penicillin biosynthesis, siderophore biosynthesis and Li^+ tolerance. The *F. oxysporum* f. sp. *lycopersici* orthologue of the *A. nidulans* PacC protein regulates expression of both alkaline and acidic condition-responsive genes (Caracuel et al. 2003). The *PACC* gene itself is overexpressed under alkaline conditions and a loss-of-function mutation in *PACC* resulted in poor growth under alkaline condition, increased acid protease activity and overexpression of acid-induced polygalacturonase genes. In contrast, a gain-of-function mutation mimicked growth under alkaline conditions with increased alkaline protease activity and acid phosphatase levels (Caracuel et al. 2003). The *PACC* gene also negatively regulates pathogenesis and a *F. oxysporum* f. sp. *lycopersici* mutant

carrying a dominant activating allele was less virulent on tomato plants than the wild-type while loss of *PACC* function resulted in earlier induction of disease symptoms (Caracuel et al. 2003). This is possibly due to negative regulation of acid-expressed genes important for infection as spore germination, root attachment, colonisation of vascular tissue and invasive growth was unaffected for both loss- and gain-of-function mutants. In *F. graminearum*, production of trichothecene mycotoxins is induced by low pH and is negatively regulated by PacC (Gardiner et al. 2009; Merhej et al. 2012).

7.4 Signalling Pathways Involved in Pathogenesis

The environmental cues are widely perceived through membrane receptors and translated into signalling response pathways through the action of G-proteins (Bölker 1998; Hamm 1998; Marinissen and Gutkind 2001). G-proteins are heterotrimeric proteins of α, β and γ subunits. In the inactive state, the Gα subunit binds GDP and, upon perception of stimuli by G-protein coupled receptors, exchanges its bound GDP for GTP and dissociates from the Gβγ subunit. The free Gα:GTP and Gβγ subunits then activate distinct downstream effectors including enzymes (adenylyl cyclase, phospholipases, phosphodiesterases and protein kinases), ion channels and small GTPases, thus regulating multiple signalling pathways and playing crucial roles in vegetative growth, sporulation, mating and virulence in fungi (Bölker 1998).

Fungal Gα proteins are grouped into three classes. The gene *FGA1* encoding α subunit of class I G-proteins has been cloned and characterised in *F. oxysporum* f. sp. *cucumerium* (Jain et al. 2002). Disruption of this gene had no appreciable effects on hyphal growth, but colony morphology was altered and the *FGA1* mutants produced approximately half the number of conidia as compared to the wild type. When cucumber plants were challenged, disease development and severity of the disease was significantly delayed in the case of the disruption mutant as compared to the wild type. Cyclic AMP (cAMP) level was also reduced in the mutants to approximately 65 % of the wild type which suggests that Gα protein of *F. oxysporum*, unlike its mammalian counterpart, positively controls cAMP levels (Jain et al. 2002). In comparison, disruption of the α protein subunit of class III G-protein (Fga2) resulted in complete loss of pathogenicity, but growth, colony morphology and conidiation was unaltered (Jain et al. 2005). The *FGA1*/*FGA2* double mutants had phenotype characteristics of either *FGA1* or *FGA2* mutants, except heat survival of bud cells in double mutants was significantly increased over the single mutants which were more resistant than the wild type (Jain et al. 2002, 2005). These observations suggest presence of two different signalling pathways for Fga1 and Fga2 possibly involving cAMP. When the gene for G-protein β-subunit (Fgb1) was disrupted, the mutant showed characteristics similar to the *FGA1* disruption mutant (Jain et al. 2003). In the mutant, hyphal growth remained unaltered, colony morphology was affected, conidia production was reduced, heat resistance was better and both cellular cAMP

and pathogenicity were reduced. While this shows that Fga1 and Fgb1 have partially overlapping functions and both positively regulate cAMP homeostasis, addition of extracellular cAMP could not restore phenotypic effects observed in the case of the *FGB1* mutant suggesting participation of Fgb1 in more than one signalling pathways that are not regulated by cAMP (Jain et al. 2003). The *FGB1* gene has also been studied in *F. oxysporum* f. sp. *lycopersici* where it regulates polarised hyphal growth, hyphal branching, microconidiation as well as virulence on tomato plants and fruits (Delgado-Jarana et al. 2005). However, in this instance, two of the altered phenotypes, hyper-elongation and reduced subapical branching could be reversed by addition of cAMP.

Another well-conserved signalling pathway that plays critical role in plant pathogenesis and infection-related morphogenesis in fungi is mitogen-activated protein kinase (MAPK) pathway. MAPKs are a family of serine/threonine kinases that are activated in response to environmental stimuli through a cascade of kinases (MAPK kinase and MAPK kinase kinase) eventually resulting in regulation of downstream transcription factors and target gene expression. In *F. oxysporum* the *FMK1* gene, encoding a member of the YERK1 (yeast extracellular signal-regulated kinase 1) MAPK family, controls several key steps in pathogenesis (Di Pietro et al. 2001, 2003). A disruption mutant of the *FMK1* gene in *F. oxysporum* f. sp. *lycopersici* failed to infect tomato plants and could not macerate tomato fruit tissue when microconidial suspension was injected into fruit tissue (Di Pietro et al. 2001). Expression of the pectate lyase genes was severely compromised in the *FMK1* mutant. In contrast, expression of endo- or exo-polygalacturonase genes was hardly affected, and hyphal growth or conidiation on potato dextrose agar in the mutant was similar to the wild type. On further examination, it was found that conidia from the *FMK1* mutant failed to germinate into germ tubes and attachment to root surface could not be established. Fmk1 is also needed for fungal hyphal fusion (Prados Rosales and Di Pietro 2008). However, phosphorylation status of Fmk1 remains unaltered in the *FGB1* mutant, and the *FGB1* mutant shows reduced virulence, while the *FMK1* mutant is totally avirulent suggesting these two proteins function in independent pathways. How these signalling cascades regulate virulence is not clear.

One upstream protein of the MAPK pathway is the transmembrane protein Msb2 which carries a large extracellular glycosylated mucin homology domain and a short cytoplasmic region (Cullen et al. 2004; Perez-Nadales and Di Pietro 2011). Msb2 is expressed at high levels at early stages of infection and regulates expression of two genes, *FRP1* and a class V chitin synthase gene that are transcriptionally activated by the MAPK cascade (Perez-Nadales and Di Pietro 2011). Msb2 is also required for contact-induced phosphorylation of the MAPK Fmk1. The Δ*msb2* gene knock-out mutants showed impaired root penetration, pectinolytic activity and invasive growth on tomato fruits, but at lesser extent, than the Δ*fmk1* or the Δ*msb2*Δ*fmk2* double mutants which suggests Msb2 contributes to Fmk1-mediated pathogenesis (Perez-Nadales and Di Pietro 2011). Msb2 also contributes additively to Fmk1 regulation of hyphal growth under nitrogen starvation conditions but does not affect hyphal fusion (Perez-Nadales and Di Pietro 2011). These observations indicate that Msb2 may not act exclusively as upstream component of the Fmk1

MAPK cascade or other upstream components exists and there is significant crosstalk amongst those components and MAPK pathways. Further research will be required to define role of Msb2 in signalling through various MAPK cascades, signalling partners as well as factors downstream of MAPK pathways that regulate pathogenesis in *F. oxysporum*.

In yeast, the homeodomain transcription factor Ste12 functions downstream of the MAPKs Fus3 and Kss1 (Rispail and Di Pietro 2009, 2010a). The Ste12 protein is activated by Fus3 and Kss1 and controls mating, filamentous and invasive growth. The *F. oxysporum* f. sp. *lycopersici* homologue of Ste12 is required for surface penetration (judged by observation of development on cellophane membranes), invasive growth on tomato fruit tissue and disease incidence in whole plants. It is not, however, essential for hyphal fusion, surface adhesion on plant roots or expression of pectinolytic enzymes (Rispail and Di Pietro 2009). This gene is upregulated in planta at early stages of infection in highly virulent strains of *F. oxysporum* f. sp. *phaseoli* as well as by nitrogen starvation. However, in contrast to *F. oxysporum* f. sp. *lycopersici*, disruption of the *STE12* gene in *F. oxysporum* f. sp. *phaseoli* only delayed development of disease symptoms, but not severity. It has been proposed that difference in organic and inorganic nitrogen status within the xylem vessels of legume and the non-legume plant system under study may account for this difference.

The other signalling gene that has been implicated in virulence in *F. oxysporum* f. sp. *lycopersici* is a two-component histidine kinase (Rispail and Di Pietro 2010b). In yeast, histidine kinase Sln1 acts as a sensor protein upstream of the osmoregulator MAPK Hog1 (Rispail and Di Pietro 2010a). The *F. oxysporum* orthologue of the *SLN1* gene, *FHK1*, is similarly required for adaptation to hyperosmotic stress (Rispail and Di Pietro 2010b). The Δ*fhk1* mutant is more sensitive to oxidative stress and demonstrated significant delay in symptom development as compared to the wild type. However unlike Δ*fmk1* or Δ*fmk1*Δ*fhk1* double mutant, deletion of only the *FHK1* gene did not impair cellophane penetration or colonisation of host tissue in the mutant. This suggests that Fhk1 contributes to plant virulence independent of Fmk1 (Rispail and Di Pietro 2010b). Opposing roles of Fmk1 and Fhk1 in response to oxidative stress and additive roles in response to hyperosmotic stress were also noted in this study. It is possible that MAPK pathways in *F. oxysporum* are involved in more than one response pathway and they possibly crosstalk.

Nutrient status of the surrounding environment is also a crucial factor in a pathogenic life style, and nutrient stress plays a crucial role in pathogenicity. In particular, nitrogen limitation has often been linked with expression of pathogenicity genes, in a number of fungi (Bolton and Thomma 2008; Snoeijers et al. 2000). Generally ammonium nitrogen is preferred as a source of nitrogen over less available sources like nitrate. The nature of available nitrogen regulates nitrogen assimilation through nitrogen catabolite repression. Genes needed for nitrogen utilisation are downregulated in the presence of a preferred source. This control is achieved through a GATA-binding zinc-finger transcription factor, AreA in *Aspergillus nidulans* and Nit2 in *Neurospora crassa*, which functions as a transcriptional

activator of genes involved in uptake and utilisation of secondary nitrogen sources (Schure et al. 2006). In response to ammonium, AreA transcription is downregulated and also its co-repressor NmrA binds to AreA to alter its DNA-binding activity, resulting in nitrogen catabolite repression. In *F. oxysporum* f. sp. *lycopersici*, the AreA orthologue Fnr1 is present at a basal level in hyphae growing on nitrate and is overexpressed when ammonia or glutamine is the source of nitrogen (Divon et al. 2006). Growth of *FNR1* gene-disruption mutants was restricted on nitrate-containing media, and their ability to utilise a number of secondary sources of nitrogen was impaired (Divon et al. 2006). In the *FNR1* disruption mutant, transcripts of genes that are involved in in planta acquisition of nitrogen, e.g. general amino acid permease (*GAP1*), peptide transporter (*MTD1*) and uricase, were detected at a lower level. Moreover, nitrate-induced expression of nitrate reductase (*NIT1*) and nitrite reductase (*NII1*) genes was not detected in the Δ*fnr1*/*AREA* null mutant, which suggests that Fnr1/AreA activates nitrogen catabolism genes in *F. oxysporum* f. sp. *lycopersici* (Divon et al. 2006; Lopez-Berges et al. 2010).

Apart from its role as transcriptional activator of nitrogen catabolism genes, Fnr1/AreA is also involved in ammonium suppression of plant infection by *F. oxysporum*. A delay in symptom development was observed in *F. oxysporum*-infected plants growing on ammonium nitrate, but not sodium nitrate, suggesting ammonium negatively regulates plant infection (Lopez-Berges et al. 2010). Ammonium also negatively regulates various infection-related phenomena like cellophane penetration, fusion of vegetative hyphae or root adhesion. This ammonium suppression of development leading to plant infection is mediated through MeaB protein in *A. nidulans* (Wong et al. 2007). MeaB is a negative regulator of AreA and functions by activating transcription of the AreA co-suppressor protein NmrA. However, contrary to what might be expected, this suppression in *F. oxysporum* f. sp. *lycopersici* is not achieved through Fnr1/AreA. Instead, Fnr1/AreA acts as an independent suppressor of cellophane penetration, hyphal fusion and root adhesion (Lopez-Berges et al. 2010). Ammonium suppression of the plant infection was only partially relieved in the Δ*fnr1*/*AREA* mutant. Fnr1/AreA is also required for ammonium-driven downregulation of *STE12* expression. Together these observations strongly suggest that Fnr1/AreA in *F. oxysporum* f. sp. *lycopersici* serves a dual role; it acts as an activator of nitrogen catabolism genes and as a repressor of nitrogen-regulated virulence functions (Lopez-Berges et al. 2010). A similar loss of severity of disease symptoms and lower expression of pathogenicity genes have been reported in AreA/Nut1^{-} mutant in *Magnaporthe grisea* (Soanes et al. 2002). In *Cladosporium fulvum*, however, disruption of the GATA-binding nitrogen response factor gene *NRF1* did not affect virulence (Pérez-García et al. 2001).

Another protein that is involved in nitrogen regulation of virulence functions in *F. oxysporum* is the serine/threonine kinase TOR (target of rapamycin). TOR is a key player in the nutrient response pathway in *Saccharomyces cerevisiae*, and inhibition of TOR by rapamycin mimics nitrogen stress. In *F. fujikuroi*, blocking TOR function resulted in perturbation of expression of number of AreA-regulated

genes (Teichert et al. 2006). TOR inhibition in *F. oxysporum* f. sp. *lycopersici* resulted in reversion of glutamine-induced suppression of cellophane penetration, hyphal fusion, root adhesion and de-repression of nitrogen catabolism genes (Lopez-Berges et al. 2010). Role of TOR in nitrogen catabolite repression is not dependent on MeaB but possibly exerted through inhibition of Fnr1/AreA.

7.5 Breaking Barriers at the Gate

While invading plants, pathogenic fungi must breach through physical barriers like the plant cell wall. Primary components of plant cell walls are polysaccharides like cellulose, hemicellulose and pectin; pectic (homogalacturonan and rhamnogalacturonan) and hemicellulosic polysaccharides (xyloglucans, glucomannans, xylans and mixed-linkage glucans) form a matrix. The matrix embeds cellulose microfibrils as well as number of cell-wall proteins. While fungi like *M. oryzae* and *Ustilago maydis* use specialised structures like appressoria and penetration pegs to puncture through (Tucker and Talbot 2001), lack of such specialised structures in *F. oxysporum* has always been an important factor in linking penetration with plant cell-wall-degrading enzymes. Enzymes like exo- and endopolygalacturonases, endo-xylanase and pectate lyase have been purified, characterised, cloned and their roles in plant pathogenesis studied (Di Pietro et al. 2003; Di Pietro and Roncero 1996a, b, 1998, 2006; Garcıća Maceira et al. 1997, 2001; Gomez-Gomez et al. 2001, 2002). A general theme that came out of these studies was that disruption of the CWDE genes individually did not have any detectable effect on virulence, possibly because of functional redundancy (Di Pietro et al. 2003; Michielse and Rep 2009). Many of these CWDE genes are regulated in a concerted manner and are subjected to glucose catabolite repression. For example, the xylanase encoding genes *XYL2*, *XYL3*, *XYL4* and *XYL5* were induced in presence of oat-spelt xylan and tomato vascular tissue but were suppressed by simple sugars like glucose, xylose or arabinose in the media (Gomez-Gomez et al. 2001, 2002). A transcription factor that acts as a common positive regulator of xylanase genes, XlnR, has been identified in *F. oxysporum* f. sp. *lycopersici* (Calero-Nieto et al. 2007, 2008). A null mutant of the XlnR regulator was impaired in expression of all the three (*XYL2*, *XYL3* and *XYL4*) genes, but disease induction on tomato plant was not affected (Calero-Nieto et al. 2007). However, this mutant was able to grow on xylan as sole carbon source indicating either residual xylanase activity is sufficient for breaking down plant cell walls or there could be yet undiscovered xylanase genes.

The *SNF1* (sucrose non-fermenting) gene, which codes for a protein kinase, is essential for de-repression of a battery of genes and invasive growth repressed by glucose in *S. cerevisiae* (Palecek et al. 2002; Young et al. 2012). A mutant of the *SNF1* gene in *F. oxysporum* exhibited restricted growth on complex carbon sources and expression of exo- and endopolygalacturonase and pectate lyase genes was substantially lower than in the wild-type pathogen under de-repressive conditions

(Ospina-Giraldo et al. 2003). The *SNF1* mutants were also less virulent on *A. thaliana* and cabbage plants, possibly as a result of less effective cell-wall penetration. However, this could also be due to impaired ability of the mutants to grow on complex carbon sources as symptom development was also delayed on plants where roots were cut to facilitate entry.

Two other proteins that play a role in glucose repression of CDWE are Frp1 and Cre1 (Jonkers and Rep 2009a; Jonkers et al. 2009). The Frp1 protein belongs to the family of F-box proteins that form the SCF (Skp1–Cullin–F-box protein) complex involved in ubiquitination and subsequent degradation of target proteins (Jonkers and Rep 2009b). F-box proteins bind specific substrates destined for ubiquitination and link them to the SCF complex by binding Skp1 through their F-box motifs. The Frp1 protein was first identified as a protein required for pathogenesis in *F. oxysporum* f. sp. *lycopersici* through insertional mutagenesis experiments (Duyvesteijn et al. 2005). Reduced virulence of the Δ*frp1* mutant on tomato plants was related to its inability to penetrate tomato roots possibly due to reduced expression of several CWDE genes including *XYL2*, *XYL3*, *XYL5*, *PG1* (endo-polygalacturonase), *PL1* (pectate lyase) and *ARA1* (arabinase) (Jonkers et al. 2009). Interestingly, expression of the XlnR regulator protein was not affected in the mutant, suggesting that deletion of the Frp1 protein resulted in permanent repression of the CWDE genes. The Δ*frp1* mutant also failed to grow normally on a number of complex sugar sources like organic acids, sugar alcohols and polysaccharides. These growth defects are attributed to severely reduced expression of the isocitrate lyase enzyme (*ICL1*) (Jonkers et al. 2009).

Attempts to generate a Δ*cre*A knock-out mutant in wild type or in Δ*frp*1 background was not successful, but when a N-terminal GST fusion with CreA replaced wild-type CreA, the mutants behaved similar to Δ*cre*A mutants in *Aspergillus nidulans* and *Neurospora crassa* and wild-type colony morphology could be restored by ectopic overexpression of wild-type CreA in the GST:CreA mutants (Jonkers and Rep 2009a). Expression of the *ICL1*, *ARA1* and *XYL2* genes was restored in this GST:CreA mutant in the Δ*frp1* background (Jonkers and Rep 2009a). Accompanying these changes, the ability to grow on complex sugar sources and infect plants was also restored in the ΔFrp1/GST:CreA mutant. In *A. nidulans* a number of genes expressed during growth on glucose and ethanol and regulated by CreA and Ara1, and *XLY2* genes from *F. oxysporum* contain potential CreA binding sites in their promoter regions (Jonkers and Rep 2009a). How Frp1 and CreA function together in regulating carbon catabolite repression is currently unclear.

While the fungus tries to breach through the plant cell wall using the CWDEs, integrity of its own cell wall is also equally important for successful invasion. An important structural component of cell wall of filamentous fungi is chitin. Chitin synthases which catalyse chitin chain formation are integral membrane proteins that play essential roles in hyphal growth and differentiation (Roncero 2002). There are several structural classes of chitin synthase, and representatives of classes I–V and VII have been identified in *F. oxysporum* f. sp. *lycopersici* (Madrid et al. 2003; Martin-Udiroz et al. 2004, 2008). Targeted disruption of the *CHSV* gene rendered the fungus sensitive to tomato root extracts and in particular to the plant's defence

compounds H_2O_2 and α-tomatine (Madrid et al. 2003). The mutant exhibited some evidence of hyphal lysis and balloon-like swellings that are densely stained by the chitin-binding dye calcofluor white. These abnormalities could be reversed by addition of osmoprotectant to the growth media. All of these observations strongly indicate that the cell-wall integrity of the fungus is affected in the deletion mutant. Similar defects in cell-wall structure were documented in the gene-deletion mutant of the *CHSVb* gene, a class VII chitin synthase, although unlike the Δ*chsV* mutant some of these effects could not be reversed by addition of sorbitol into media (Martinez-Rocha et al. 2008). Also, inter-hyphal septa were observed in the Δ*chsVb*, but not the Δ*chsV* mutant indicating *CHSV* and *CHSVb* play somewhat different roles in hyphal morphogenesis. Both of these gene-disruption mutants were severely compromised in plant infection. Plants inoculated with these mutants did not show any wilt symptoms and remained as healthy as mock-inoculated plants (Madrid et al. 2003; Martinez-Rocha et al. 2008). Null mutants of the *CHS1* and *CHS7* genes were reduced in virulence and were more sensitive to SDS, but hyphal lysis, or sensitivity towards calcofluor white were like wild-type strains (Martin-Udiroz et al. 2004). An association of chitin synthases and virulence in plants is well documented in fungal pathosystems (Lenardon et al. 2010). Disrupted cell-wall structures may lead to early leakage of fungal cell components triggering an early defence response from plants and/or render these mutants more susceptible to plant antimicrobial defence compounds. Interestingly, promoter regions of the *CHS2*, *CHS3*, *CHS7* and *CHSV* genes have a stress-response element binding site. It is possible that chitin synthase genes function to maintain cell-wall integrity in response to plant defence compounds and are thus an important part of pathogenesis (Madrid et al. 2003; Martin-Udiroz et al. 2004, 2008). In line with this suggestion, it was observed that deletion of the *F. oxysporum* f. sp. *lycopersici gas1* gene encoding the glucan-processing enzyme, β-1,3-glucanosyl transferase, resulted in substantially reduced virulence towards tomato plants(Caracuel et al. 2005). However, at the same time, the deletion mutants were more resistant to cell-wall-degrading enzymes. The null mutant also exhibited increased expression of *CHSV* and a Rho-type GTPase gene that acts as a master regulator of cell-wall integrity (Caracuel et al. 2005; Levin 2005). The exact role of *GAS1* in pathogenesis remains to be determined. Loss of virulence in the null mutants can be explained by possible defects in host surface recognition, hyphal morphogenesis or increased secretion of potent plant defence elicitors like β-1,3-glucan- and β-1,6-glucan-protein complexes as reported for *GAS1* deletion mutants in yeast (Kapteyn et al. 1997). Targeted disruption of the *RHO1* gene in *F. oxysporum* also resulted in alterations of cell-wall structures, changes in chitin and glucan synthase enzymatic activities and reduced virulence on tomato plants (Martinez-Rocha et al. 2008). In summary all these observations underline the importance of fungal cell-wall architecture and morphogenesis in plant infection.

7.6 Coping with Plant Defence Compounds

Apart from the structural barriers to pathogen entry, plants employ variety of secondary metabolites and pathogenesis-related proteins many of which have antifungal activities. One such secondary metabolite present in tomato plants is α-tomatine, a glycosylated terpenoid phytotoxin with soap-like property (saponins) that can induce apoptotic cell death in *F. oxysporum* f. sp. *lycopersici* (Ito et al. 2007). *Fusarium oxysporum*, like many other pathogenic fungi, detoxifies this terpenoid glycoalkaloid using the glycosyl hydrolase enzyme tomatinase (Lairini et al. 1996), and hydrolysis products can suppress induced defence responses in the plant (Ito et al. 2004). One tomatinase gene *TOM1* encoding a family 10 glycosyl hydrolase enzyme has been cloned and characterised from *F. oxysporum* f. sp. l*ycopersici* (Roldan-Arjona et al. 1999). This gene is induced by tomatine and expressed in planta throughout the disease cycle (Roldan-Arjona et al. 1999). Partial silencing of this gene in *F. oxysporum* f. sp. *lycopersici* resulted in reduced virulence on tomato (Ito et al. 2002). On solid media containing α-tomatine, growth of a Δ*tom1* mutant was reduced as compared to wild type, and the null mutant was also delayed in symptom development (Pareja-Jaime et al. 2008) confirming tomatinase activity is required for full virulence. In contrast, symptom development and death were hastened for plants infected with a constitutively *TOM1* overexpressing line (Pareja-Jaime et al. 2008). However, some residual tomatinase activity was detected in the Δ*tom1* null mutant. This is possibly due to presence of four more putative tomatinase genes with high homology to glycosyl hydrolase family 3 genes in *F. oxysporum* f. sp. *lycopersici*, two of which, *Tom2* and *Tom5*, are expressed in the null mutant (Pareja-Jaime et al. 2008).

The other class of chemicals plants employ in their defence are phenolic compounds like flavonoids, anthocyanins, phytoalexins, tannins, lignin and furanocoumarins. While phenolic compounds can act as precursors of physical defence system (e.g. lignification) or antimicrobial/antifungal agents, pathogens can not only detoxify them but also can exploit these chemicals as cues for host recognition and/or source of nutrients. One way for *F. oxysporum* to deal with plant phenolics is oxidising them through the action of laccase, a copper-containing enzyme that has been implicated in morphogenesis, interaction with host, defence and lignin degradation in fungi (Thurston 1994). So far, six laccase genes, *LCC1–5* and *LCC9* have been isolated from *F. oxysporum* f. sp. *lycopersici* (Canero and Roncero 2008a). Of these, expression of *LCC3* and *LCC5* is pH dependent and appears to be regulated by the pH-responsive transcription factor PacC, whereas *LCC1*, *LCC3* and *LCC9* are differentially expressed during plant infection. However, although disruption mutants of the *LCC1*, *LCC3* and *LCC5* genes were more sensitive to oxidative stress and phenolic compounds that can act as defence chemicals and/or lignin precursors, they were as pathogenic as the wild type (mutants for *LCC2*, *LCC4* and *LCC9* could not be generated) (Canero and Roncero 2008a). In contrast, a mutant of the chloride channel gene *CLC1* was more sensitive to oxidative stress, had reduced laccase activity and was delayed in disease

symptom development (Canero and Roncero 2008b). This could be due to requirement of the chloride channel for effective loading of copper, an allosteric effector of laccase enzymes. Thus, while not essential individually, cumulative laccase activity may contribute towards fungal virulence.

Lignin breakdown products are further metabolised by soil microbes through the β-ketoadipate pathway (Harwood and Parales 1996). Through this pathway, lignin monomers generated through oxidative depolymerisation by peroxidases and laccases along with other aromatic carbohydrates and amino aromatics are converted to protocatechuate or catechol and finally to β-ketoadipate. Additional enzymatic steps then covert β-ketoadipate into TCA cycle intermediates succinyl CoA and acetyl CoA. In a random insertional mutagenesis experiment, two genes coding for catechol dioxygenase and carboxy-cis,cis-muconate cyclase enzymes of the β-ketoadipate pathway were identified as pathogenicity genes for *F. oxysporum* f. sp. *lycopersici* (Michielse et al. 2009a). The carboxy-cis,cis-muconate cyclase (*CMLE1*) gene-deletion mutant failed to develop any wilt symptom in tomato plants verifying importance of this enzyme in plant infection (Michielse et al. 2012). This enzyme was also essential for growth on number of aromatic compounds, e.g. ferulic acid, coumaric acid, vanillic acid and cinnamic acid as well as on lignin. Coumaric, vanillic and cinnamic acid can have an inhibitory effect of growth and germination of *F. oxysporum* f. sp. *niveum* (Wu et al. 2008a, b, c), and coumaric acid at high doses of 100 mg/L could reduce germination frequency in the Δ*cmle1* mutant of *F. oxysporum* f. sp. *lycopersici* (Michielse et al. 2012). The CMLE enzyme is required for in planta pathogen growth, but not for spore germination and root colonisation. This implies that the loss of pathogenicity in the Δ*cmle1* mutant could be due its inability to catabolise lignin and its breakdown products. Toxic effects of various plant phenolics could also contribute to this loss of pathogenicity in this mutant.

7.7 Fighting Back: Toxin Production

Fusarium species produce an array of secondary metabolites with equally diverse mode of biosynthesis and function. Many of these secondary metabolites are toxic to other organisms. In particular, fumonisins and trichothecenes are of great concern due to their presence in *Fusarium*-infected cereals. While *F. verticillioides* and *F. graminearum* gained worldwide importance as producers of these two mycotoxins, *F. oxysporum* also produces number of secondary metabolites (Luz et al. 1990), and the *F. oxysporum* f. sp. *lycopersici* genome contains 12 clusters of genes predicted to be involved in secondary metabolite production (Ma et al. 2010). While information on the role of individual metabolites or genes is limiting, *F. oxysporum* culture filtrates and in particular fusaric acid have been extensively used to screen for plants resistant to *Fusarium* wilt (Bulk 1991; Matsumoto et al. 1995; Vu et al. 2004).

In fungi, the velvet family of regulatory proteins regulate secondary metabolite production and coordinate it with light response and morphological differentiation (Bayram and Braus 2012). Members of the velvet family interact with each other and also with LaeA, a regulator protein of secondary metabolism (Bayram and Braus 2012; Bayram et al. 2008). In *F. oxysporum* f. sp. *lycopersici*, the first clue to involvement of the velvet complex came through transposon mutagenesis experiments. A loss-of-virulence mutant in which a transposon element was inserted in a gene with homology to VelB of *A. nidulans* was identified (Lopez-Berges et al. 2009). *F. oxysporum f. sp. lycopersici* homologues of *veA*, *velC* and *laeA* have been isolated and demonstrated to have regulatory roles in hyphal development, conidiation and light response (Lopez-Berges et al. 2013). The velvet complex also regulates transcription of two nonribosomal peptide synthetase genes involved in production of the siderophores ferricrocin and triacetyl fusarinine C, two genes for biosynthesis of the mycotoxin beauvericin and an ABC transporter (Lopez-Berges et al. 2013). Concordant with their roles, production of beauvericin was reduced in the deletion mutants of the *VEA*, *VELB* and *LAEA* genes. The *VEA* and *VELB* genes, but not *VELC*, also contribute towards virulence towards tomato plants as significantly lesser number of plants died when challenged with Δ*veA* and Δ*velB* mutants, whereas the Δ*velC* mutant was as virulent as the wild type. The Δ*laeA* mutant was even less virulent and was affected in colonisation of stems at later stages of infection. VeA and LaeA are also required for successful infection of immunodepressed mice. This reduced pathogenicity on both plant and mammalian hosts was attributed to attenuated production of the mycotoxin beauvericin (Lopez-Berges et al. 2013).

7.8 Effector Proteins

One of the most exciting recent developments in the field of *Fusarium* wilt research has been the discovery of disease effector proteins produced by *Fusarium oxysporum*. Effectors are small proteins or molecules that alter host cells and function to promote pathogen growth. They may or may not elicit host-defence responses (Ellis et al. 2009; Hogenhout et al. 2009). At times an effector protein is recognised by corresponding plant resistance proteins and triggers defence mechanisms in plants. Such effectors are classically defined as avirulence proteins (Hogenhout et al. 2009). While effector proteins have been detected in a number of forma specialis of *Fusarium oxysporum*, their role in virulence to plants has only been demonstrated for a limited few.

Fusarium effectors were first described in the model wilt pathogen *F. oxysporum* f. sp. *lycopersici* as proteins secreted in the xylem (SIX) of infected tomato plants (Rep et al. 2004). So far, 11 SIX proteins have been described (Houterman et al. 2008; Lievens et al. 2009; Rep et al. 2004; Takken and Rep 2010), and the number is likely to increase (Martijn Rep, personal communication). SIX1, the founder member of these secreted proteins, is a small cysteine-rich protein that is

required for full virulence towards tomato (Rep et al. 2004). SIX1 is expressed specifically at early stages of infection, and using a GFP reporter gene fused with the *SIX1* promoter, it was demonstrated that the *SIX1* promoter is specifically induced upon entry into roots and only on living, but not dead plant material (van der Does et al. 2008). This induction was also observed in the presence of cultured cells as well as non-host cells, although only a fraction of the hyphae expressed GFP under these conditions. In tomato, the SIX1 protein is recognised by the I-3 resistance protein. However, absence or mutations in the *SIX1* gene do not always correlate with virulence on *I-3* tomato plants and led to the idea that I-3/SIX1 (Avr3)-dependent resistance can be suppressed by other effector proteins (Rep et al. 2005). Such a suppressive function is indeed played by SIX4/Avr1 protein which is present only in race 1 isolates and recognised by the tomato I and I-1 resistance proteins (Houterman et al. 2008). SIX4/Avr1 can also suppress the tomato *I-2* gene-mediated resistance, but is not needed for full virulence. The *I-2* gene-mediated resistance is triggered in response to the SIX3/Avr2 effector protein from *F. oxysporum* f. sp. *lycopersici* race 2 (Houterman et al. 2009). SIX3/Avr2 is also required for full virulence on susceptible tomato plants (lack *I-2* genes), and functional mutants of the SIX3/Avr2 proteins can evade their detection by I-2, thus breaking the I-2-mediated resistance. This deployment of various resistance genes by tomato and emergence of strategies in *F. oxysporum* f. sp. *lycopersici* for continued virulence on tomato fits well into the current zigzag model of plant-pathogen co-evolution (Jones and Dangl 2006; Takken and Rep 2010).

Discovery of these effector proteins in *F. oxysporum* f. sp. *lycopersici* prompted search for similar effectors in other formae speciales of *Fusarium*. Among the SIX proteins tested so far, SIX6 is the most widely distributed and was found in *F. oxysporum* f. sp. *vasinfectum* (cotton wilt), *F. oxysporum* f. sp. *niveum* (watermelon wilt), *F. oxysporum* f. sp. *radicis-cucumerinum* (crown and root rot of cucurbits), *F. oxysporum* f. sp. *melonis* (muskmelon wilt) and *F. oxysporum* f. sp. *passiflorae* (passion fruit wilt) among the 26 formae speciales screened in two different studies (Chakrabarti et al. 2011; Lievens et al. 2009). In *F. oxysporum* f. sp. *vasinfectum*, the *SIX6* gene was detected in pathogenic isolates from two different VCGs but was absent from closely related non-pathogenic *F. oxysporum* isolates collected from cultivated cotton fields (Chakrabarti et al. 2011). This wide occurrence, along with loss of virulence in Δ*six6* mutants of *F. oxysporum* f. sp. *lycopersici* (Martijn Rep, personal communication), suggests a general role for SIX6 in pathogenesis. Paradoxically, SIX6 was detected only in the Australian isolates of *Fov* and was absent from all the non-Australian isolates tested (Chakrabarti et al. 2011). It is possible that Australian isolates of *F. oxysporum* f. sp. *vasinfectum*, which are phylogenetically distinct from the overseas isolates, uniquely employ SIX6 in infecting the cotton plants (Chakrabarti et al. 2011).

SIX6 also shows an extraordinary pattern of sequence variation between different formae speciales. All of the SIX6 proteins are 214/215 amino acids long and have conserved cysteine residues most likely involved in disulphide-bond formation (Chakrabarti et al. 2011). Most of the *Fov*-SIX6 protein shows high sequence identity with the other SIX6 proteins, but there is a 48-amino-acid region of high

sequence divergence (only 35 % sequence identity) which includes a stretch of 14 amino acids with no sequence identity to *Fol*-SIX6. On the N-terminal side of this 48-amino-acid region, *Fov*-SIX6 shows complete identity with *Fol*-SIX6 and an 89 % identity on the C-terminal side. In contrast to *Fov*-SIX6, the SIX6 proteins from other subspecies have fewer variant residues compared to *Fol*-SIX6, and these are more evenly distributed. It is likely that the conserved cysteine residues are important for structural integrity while intervening residues are involved in effector function and have evolved to avoid detection or acquire a novel virulence function. Intriguingly, the 14 divergent residues in *Fov*-SIX6 are flanked by cysteine residues and so probably form a variant protein loop anchored at either end by disulphide bonds. The *Fov*-SIX6 and *Fol*-SIX6 proteins therefore offer a unique opportunity to compare both protein structure and function in different pathosystems.

Recently SIX1, SIX4, SIX8 and SIX9 homologues have been detected in the genome of the *Arabidopsis*-infecting *F. oxysporum* isolate *Fo*5176 (Thatcher et al. 2012). Conservation at the amino acid sequence level between the *F. oxysporum* f. sp. *lycopersici* effectors and their *Fo*5167 counterparts varied from 65 % (SIX9) to near identity (SIX4). The *Fo5176-SIX4* gene is highly expressed during plant infection, and deletion of this gene resulted in loss of virulence (Thatcher et al. 2012). A positive role of *Fo*5176-SIX4 effectors was also demonstrated by increased susceptibility of Arabidopsis plants expressing *Fo5176-SIX4*. Jasmonic acid response genes were also downregulated in the deletion mutant. Jasmonic acid response plays a significant role in wilt disease development in *Arabidopsis*, and it has been proposed that *Fusarium oxysporum* promotes jasmonate signalling to induce host susceptibility (Kidd et al. 2009; Thatcher et al. 2009). Thus, the *Fo5176*-SIX4 protein may function through activation of jasmonate signalling pathways. However, other *Fusarium* proteins must be involved as activation of the jasmonate signalling pathway was not observed in *Arabidopsis* plants expressing *Fo5176*-SIX4 (Thatcher et al. 2012).

Similar attempts were made to identify effector proteins in *F. oxysporum* f. sp. *cubense*, and genes with homology to *Fol*-SIX1 were detected in all pathogenic races of *F. oxysporum* f. sp. *cubense*, whereas SIX7 and SIX8 homologues were detected only in the race 4 isolates (Meldrum et al. 2012). However, in no case full-length genes have been isolated, nor have their functions been studied.

In the cotton *F. oxysporum* f. sp. *vasinfectum* system, microarray experiments detected more than 2,100 genes that are expressed during plant infection under tissue culture conditions (Dowd et al. 2004; McFadden et al. 2006). Bioinformatic analysis of these differentially expressed genes identified 30 genes encoding SIX-like proteins (cysteine-rich secreted proteins) for which homologues were present in the *Fusarium* database but no function was predicted (Chakrabarti et al. 2011). An additional 31 genes encoding similar cysteine-rich secreted proteins were detected for which no similar genes could be found in any databases (potentially cotton or *F. oxysporum* f. sp. *vasinfectum* genes). These 61 genes were further tested for their presence or absence in *F. oxysporum* f. sp. *vasinfectum* isolates and related non-pathogenic *F. oxysporum* isolates collected from cultivated cotton fields. Two putative effector protein genes, *PEP1* and *PEP2*, were detected only

in *F. oxysporum* f. sp. *vasinfectum* isolates, but not in the non-pathogens (Chakrabarti et al. 2011). *PEP1* encodes a 270-amino-acid protein with eight cysteines, and a homologue of *PEP1* interrupted by a transposon is present in the chromosome 14 of *F. oxysporum* f. sp. *lycopersici* (pathogenicity chromosome; see later). *PEP1* appears to be an intact expressed gene and member of a small gene family. PEP2 encodes an 86-amino-acid protein and was absent in non-Australian isolates of *F. oxysporum* f. sp. *lycopersici*. However, sequences similar to *PEP1* and *PEP2* genes could be detected in *F. oxysporum* isolates collected from native Australian *Gossypium* spp. (Chakrabarti et al. 2011). Whether PEP1 and PEP2 are true effectors and if so, their role in pathogenesis remains to be established.

7.9 Transcription Factors

Generally pathogenicity genes are defined as genes that are unnecessary for vegetative growth but required for plant infection. It is only natural that expression of the pathogenicity genes will be controlled by transcription factors in response to physical, physiological or environmental cues. There are almost 900 putative transcription factors identified in *F. oxysporum* f. sp. *lycopersici* with a preponderance of fungal specific Zn2Cys6-type transcription regulators (Ma et al. 2010). Not surprisingly, two such transcription factors, Fow2 and Sge1, were identified as pathogenicity genes in random mutagenesis experiments (Imazaki et al. 2007; Michielse et al. 2009b). Fow2 is a Zn2Cys6-type transcription factor identified in *F. oxysporum* f. sp. *melonis* as essential for plant infection but dispensable for hyphal growth on complete media, conidiation and utilisation of 95 different carbon sources (Imazaki et al. 2007). Initial stages of infection like spore germination and root attachment were unaffected in the Δ*fow2* mutant, but the mutant failed to invade root and colonise plant tissue. Homologues of Fow2 were detected in six other formae speciales of *F. oxysporum*, and disruption of Fow2 in *F. oxysporum* f. sp. *lycopersici* also resulted in similar loss of pathogenicity on tomato plants suggesting a conserved role of this transcription factor in plant infection (Imazaki et al. 2007).

FTF1 is another Zn2Cys6 transcription factor that is specifically expressed during plant infection in *F. oxysporum* f. sp. *phaseoli* (Ramos et al. 2007). The *FTF1* gene is present in multiple copies only in the highly virulent strains of this pathogen and is absent from weakly virulent or non-pathogenic isolates. Furthermore, in planta expression level of *FTF1* correlated with degree of virulence (de Vega-Bartol et al. 2011; Ramos et al. 2007). Whether FTF1 is required for plant infection or how it functions is not clear. Interestingly, in Australian isolates of *F. oxysporum* f. sp. *vasinfectum*, at least 12 copies of this gene are present, while non-pathogens isolated from cultivated cotton fields had only 3–5 copies (Chakrabarti et al. 2011).

The *SGE1* gene encodes a nuclear protein homologous to the WOR1 and RYP1 transcriptional regulators that regulate the infection-related dimorphic switch in

Candida albicans and *Histoplasma capsulatum*. A *SGE1* ortholog was initially identified in *Agrobacterium*-mediated random insertional mutagenesis experiments as a non-pathogenic mutant of *F. oxysporum* f. sp. *lycopersici* (Michielse et al. 2009a). A deletion mutant of the *SGE1* gene could successfully colonise root surface and penetrate roots but was impaired in parasitic growth within xylem tissue (Michielse et al. 2009b). The Δ*sge1* mutant also produced significantly less conidia, but conidia germination was unaffected. The *SGE1* gene also regulates expression of at least four SIX genes (*SIX1*, *SIX2*, *SIX3* and *SIX5*; Michielse et al. 2009b). *SIX1* and *SIX2* genes are not expressed in axenic culture but are induced post-penetration and/or in presence of tomato cells, while *SIX3* and *SIX5* are expressed under both conditions. Expression of *FOW2* and *FTF1* genes were also altered in the Δ*sge1* mutant, and production of fusaric acid and beauvericin was also lowered (Michielse et al. 2009b). Expression of the *SGE1* is 3–5-fold upregulated during plant infection. SGE1 may also be regulated at the post-translation level, as it carries a putative protein kinase A phosphorylation site that is required for its role in pathogenicity (Michielse et al. 2009b). Whether or how SGE1 acts as a global regulator of plant infection or how expression/function of SGE1 itself is regulated remains to be elucidated. FGP1, a WOR1-like protein from *F. graminearum*, is similarly involved in conidiation and regulates a number of genes involved in secondary metabolism including trichothecene production (Jonkers et al. 2012). However, *SGE1* could not complement *FGP1* and vice versa, and hence these two regulators, despite their sequence similarity, are functionally divergent (Jonkers et al. 2012).

HapX is a transcription factor of the bZIP family that plays a role in iron homeostasis and virulence in *F. oxysporum* f. sp. *lycopersici* (Lopez-Berges et al. 2012). Iron is an essential element for any organism, and its level is tightly regulated as increased iron levels are toxic. Loss of the *HAPX* gene resulted in reduced growth under iron-limiting conditions, but iron content in the Δ*hapX* mutant was similar to the wild type (Lopez-Berges et al. 2012). However, this also caused an increase in intercellular siderophores and de-repression of iron-regulated genes that are repressed under iron starvation. Interestingly, many genes were also induced under iron-limiting conditions in a *HAPX*-dependent manner and included number of known pathogenicity genes, e.g. glucanase, pectate lyase and *SIX3* (Lopez-Berges et al. 2012). Many iron-starvation-induced genes, e.g. *SRBA*, *SIDA* and *HAPX*, are also dramatically induced at early stages of plant infection; wilt symptom development was significantly delayed in the Δ*hapX* mutant as compared to wild type (Lopez-Berges et al. 2012). Mortality of immunocompromised mice challenged with *F. oxysporum* was also significantly lower for the Δ*hapX* mutant (Lopez-Berges et al. 2012). This shows that reprogramming of iron-dependent genes through HapX is an important aspect of both mammalian and plant infection by *F. oxysporum*. In line with this observation, both growth and plant infection by the Δ*hapX* mutant were further impaired in presence of root-colonising *Pseudomonas putida* and *P. fluorescens* (Lopez-Berges et al. 2012). The antagonistic activity of these pseudomonads was dependent on siderophore production,

suggesting that direct competition for iron is important for biocontrol by these bacteria.

7.10 Genomics of *Fusarium oxysporum*: Lineage-Specific Regions and Pathogenicity Chromosomes

The first available *F. oxysporum* genome is from the tomato pathogen *F. oxysporum* f. sp. *lycopersici* strain 4287 (NRRL 34936) and is 60 Mb in size which is significantly larger than the nearest *F. verticillioides* (42 Mb) and related *F. graminearum* (36 Mb) genomes (Cuomo et al. 2007a; Ma et al. 2010). The *F. oxysporum* f. sp. *lycopersici* genome sequence has been assembled in 15 chromosomes and contains 17,735 coding genes (Ma et al. 2010). A significant part of the genome is composed of repetitive sequences (16.8 Mb) and transposable elements (3.98 %) and accounts for much of the increased size in *F. oxysporum* f. sp. *lycopersici* genome. A three-way comparison between *F. oxysporum*, *F. verticillioides and F. graminearum* genomes identified more than 9,000 orthologous genes with high sequence similarity (an average identity of 91 % with *F. verticillioides* and 85 % with *F. graminearum* counterparts; Ma et al. 2010). When compared to other Ascomycetes, these three *Fusarium* species are enriched in genes for predicted transcription factors, lytic enzymes, transmembrane transporters and G-protein-coupled receptors (Ma et al. 2010). Altogether 2,280 transcription factors from 46 different families have been predicted in these three *Fusarium* species. Within this group, *F. oxysporum* is particularly enriched in transcription factors from the bHLH, Zn2Cys6 and bZIP families (Ma et al. 2010). All three fungi also carry the full complement of plant cell-wall-degrading enzymes in which pectate lyases are particularly over-represented due to an increase in copy number (members of the pectate lyase 1, 2 and 4 family) as well as presence of unique members (pectate lyase 9; Ma et al. 2010). Members of all the five classes of ABC transporters are also over-represented in all the three *Fusarium* species with *F. oxysporum* having the highest number of these transporters (Ma et al. 2010). This increase in number of ABC transporters is quite significant, because members of the pleiotropic drug resistance protein transporter family (or ABCG family) are responsible for tolerance to toxic compounds and plant secondary metabolites. This may explain *Fusarium* species tolerance to various azoles. Additionally there are five unique ABC transporters in *Fusarium* that are not present in any other sequenced fungal genomes. Members of the G-protein-coupled receptor family like the cAMP-receptor-like proteins, opsins/opsin-related proteins, homologues of human membrane progestin receptor and homologues of *M. oryzae* PTH11 are also over-represented in all the three *Fusarium* species (Ma et al. 2010). In particular, homologues of *PTH11*, a known pathogenicity factor in *M. grisea* (DeZwaan et al. 1999) are far more prevalent in *Fusarium* species; 98 members of this family are present in *F. oxysporum* as compared to 60 in *M. grisea*. In line with the

diversity of secondary metabolites produced by *Fusarium*, a number of secondary metabolite genes grouped into clusters was also identified in all the three *Fusarium* species (Ma et al. 2010). Genes from the majority of these clusters are co-regulated. However, only few of these secondary metabolite gene clusters are shared amongst these three species.

Genome sequences of the three *Fusarium* species were also analysed for synteny and chromosomal rearrangements (Kistler et al. 1998; Rep and Kistler 2010). Most of the *F. verticillioides* 11 chromosomes aligned in large, almost end-to-end blocks with any one of the four large chromosomes of *F. graminearum*. In addition to polymorphism-rich telomere proximal regions, *F. graminearum* chromosomes also have discrete interstitial regions of diversity. These interstitial polymorphic regions of *F. graminearum* chromosomes aligned well with ends of *F. verticillioides* chromosomes and mark sites of chromosomal fusion in *F. graminearum*.

When *F. oxysporum* and *F. verticillioides* genomes were aligned, over 90 % of the *F. verticillioides* genome was found to be syntenic to *F. oxysporum*, and all the 11 chromosomes of *F. verticillioides* had their corresponding ones in *F. oxysporum* with little local rearrangement and only one major translocation event (Ma et al. 2010). In contrast there were no significant orthologous sequences in *F. verticillioides* for the whole of chromosomes 3, 6, 14 and 15; part of chromosomes 1 and 2 and most of the unmapped regions from *F. oxysporum*. These are the lineage-specific (LS) regions in *F. oxysporum*. These lineage-specific regions of the *F. oxysporum* genome are rich in transposable elements, particularly DNA transposons, and arose through segmental duplications, with little exchange of genetic information with conserved regions of the genome (Ma et al. 2010). When short sequence reads from *F. oxysporum* strain 5176, a pathogen on *Arabidopsis*, were aligned with *F. oxysporum* f. sp. *lycopersici* genome sequences, the short alignments did not uniformly cover the genome. While conserved regions of the genome had greater than 80 % coverage, genomic regions corresponding to the lineage-specific regions of the *F. oxysporum* f. sp. *lycopersici* genome were covered at a very low level (0–10 %). Thus, the lineage-specific regions of the *F. oxysporum* f. sp. *lycopersici* genome are also enriched in strain- or forma specialis-specific sequences (Ma et al. 2010). In line with this, it was also observed that all the fungal ESTs detected in *F. oxysporum* f. sp. *vasinfectum* infected cotton plants aligned with conserved regions of the *F. oxysporum* f. sp. *lycopersici* genome.

The lineage-specific regions of the *F. oxysporum* f. sp. *lycopersici* genome are particularly enriched in secreted proteins (Ma et al. 2010; Rep and Kistler 2010). These include nine SIX proteins, several other small cysteine-rich proteins of unknown function (potential effectors), necrosis- and ethylene-inducing proteins (NEP) and carbohydrate active enzymes (CAZY). All of these proteins either have been demonstrated or are predicted to play important roles in plant pathogenesis. *F. oxysporum* f. sp. *lycopersici* genes encoded in the lineage-specific region are very similar to the NEP genes from *Verticillium dahliae*, the only other wilt-producing fungus (Klosterman et al. 2011). Among the CAZYs, glycoside hydrolases are particularly over-represented in the lineage-specific regions, and many of them are expressed during plant infection (Ma et al. 2010). This expansion

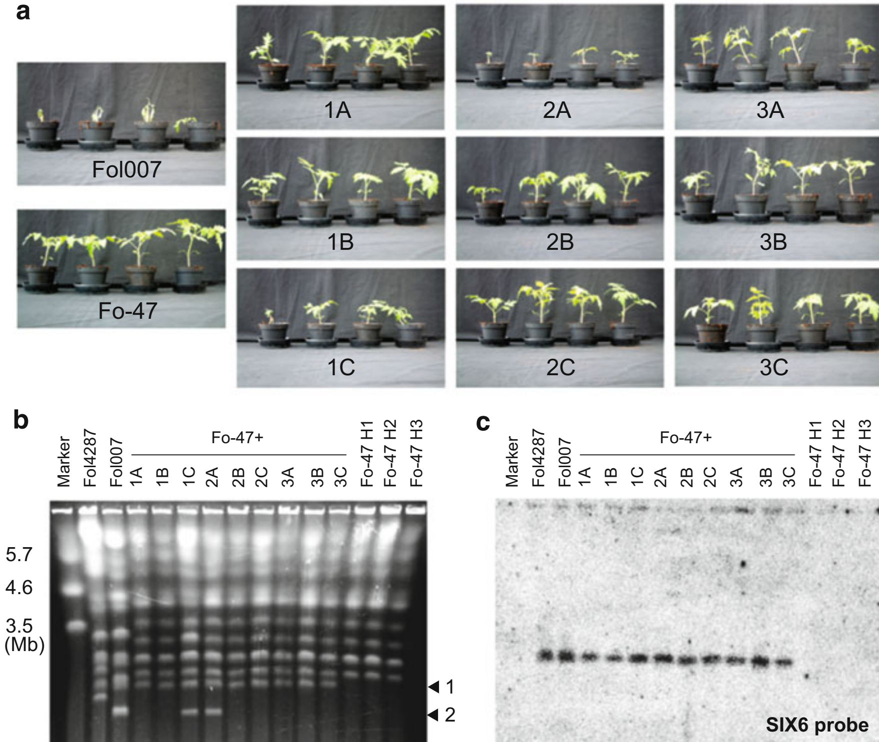

Fig. 7.2 A pathogenecity chromosome is present in *F. oxysporum* f. sp. l*ycopersici*. Antibiotic-resistant pathogenic strains (*Fol-007*), nonpathogenic strains (*Fo-47*) and double-drug-resistant strains obtained by cocultivating of *Fo-47* and *Fol-007* were tested for disease incidence on tomato (**a**). All but one of the double-drug-resistant strains were virulent (*Fo-47*+; 1A-3C). *Fol-4287* (sequenced strain), *Fol-007*, *Fo-47*+ and *Fo-47* strains (H1–H3) were karyotyped using contour-clamped homogenous electric field (CHEF) gel electrophoresis (**b**). All the *Fo-47*+ strains have the same karyotype as *Fo-47* except for the presence of one or two small chromosomes from *Fol-007* (*black arrowheads*). The larger of these two transferred chromosomes carries the *SIX6* gene present in the chromosome 14 of *Fol-4287* (**c**) and is the "pathogenicity" chromosome in *F. oxysporum* f. sp. *Lycopersici*. Adapted by permission from Macmillan Publishers Ltd: Nature (Ma et al. 2010), copyright 2010

of CWDEs may account for the broad host range of *F. oxysporum*. Other proteins that have been expanded in *F. oxysporum* are secreted peroxidases that provide protection to oxidative stress and proteins involved in lipid metabolism and generation of lipid-derived secondary messengers, GPCRs and regulator of G-proteins signalling (RGS; Ma et al. 2010). Examples include a perilipin-like Cap20 protein that regulates release of free fatty acids and is a pathogenicity determinant in *Colletotrichum gloeosporioides*. This protein is present in nine copies in *F. oxysporum* as opposed to single copies in *F. graminearum* and *F. verticillioides*.

The small non-orthologous chromosomes, chromosomes 3, 6, 14 and 15, of *F. oxysporum* f. sp. *lycopersici* are absent from a *F. oxysporum* strain

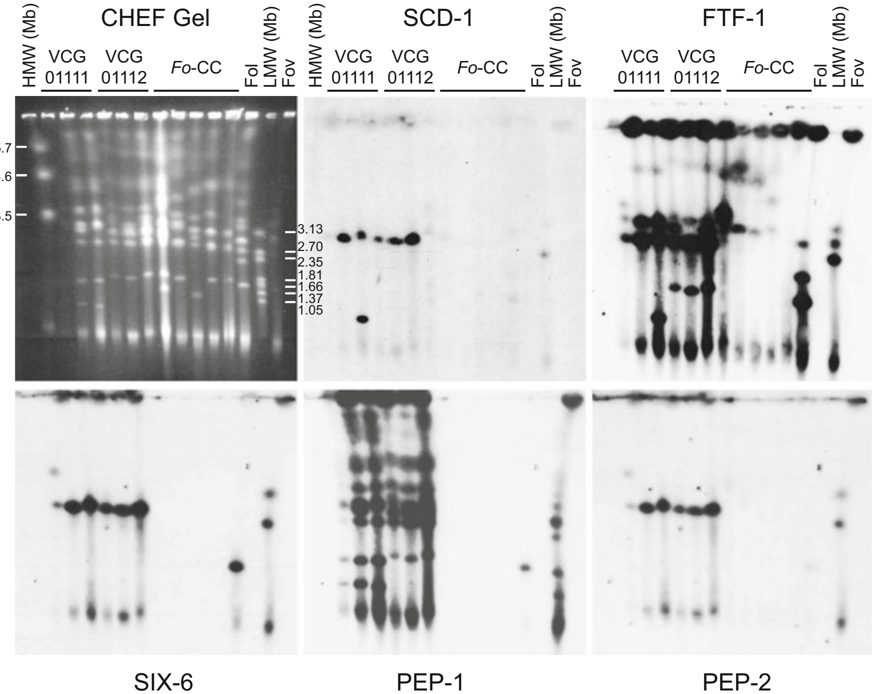

Fig. 7.3 A supernumerary chromosome is present in *F. oxysporum* f. sp. *vasinfectum*. Chromosomes from *F. oxysporum* f. sp. *vasinfectum* isolates from two different VCGs, related nonpathogenic *F. oxysporum* isolates collected from cultivated cotton fields (Fo-CC) and *F. oxysporum* f. sp. *lysopersici* (Fol) were karyotyped. The supernumerary chromosome in *F. oxysporum* f. sp. *vasinfectum* isolates carries *Fov-SIX6*, *PEP2* and at least one copy of *PEP1*, *SCD1* and *FTF1*. *Fov-SIX6*, *PEP1* and *PEP2* are present only in the pathogenic isolates, and the *FTF1* and *SCD1* genes are amplified in the pathogenic isolates. This supernumerary chromosome is an ideal candidate for pathogenicity chromosome in *F. oxysporum* f. sp. *vasinfectum* (Chakrabarti et al. 2011)

non-pathogenic on tomato (Ma et al. 2010; Rep and Kistler 2010) (Fig. 7.2). Likewise, non-conserved supernumerary chromosomes carrying genes for resistance to plant antimicrobials, utilisation of specific carbon and nitrogen sources and host-specific pathogenicity are also described in *Nectria haematococca* (anamorph *F. solani*; Coleman et al. 2009) and *Alternaria alternata* (Hu et al. 2012). A supernumerary chromosome has been described for the cotton pathogen *F. oxysporum* f. sp. *vasinfectum* (Chakrabarti et al. 2011). This ~3.6-Mb chromosome is present in *F. oxysporum* f. sp. *vasinfectum* isolates from two different VCGs but is absent from phylogenetically related non-pathogenic *F. oxysporum* isolates collected from cultivated cotton fields and carries all three putative effector genes (Fov-SIX6, PEP1 and PEP2) identified in *F. oxysporum* f. sp. *vasinfectum* and at least one copy of the *FTF1* and *SCD1* genes that are specifically amplified in *F. oxysporum* f. sp. v*asinfectum* (Fig. 7.3).

Over-representation and expansion of genes coding for potential effectors, virulence factors, CWDEs, transcription factors and other pathogenesis-related genes and a near-absence of housekeeping genes in the lineage-specific regions of the *F. oxysporum* f. sp. *lycopersici* genome combined with their specific presence in pathogenic isolates strongly suggest a connection between presence of these lineage-specific regions and plant infection as well as host range. In particular, the supernumerary chromosome 14 of *F. oxysporum* f. sp. *lycopersici* contains all the known genes for in planta secreted proteins (Ma et al. 2010; Rep and Kistler 2010). Conidia from *F. oxysporum* f sp. *lycopersici* strain 007 (*Fol*-007) with a selectable marker gene (Zeocin, BLE) inserted in the chromosome 14 was cocultured with conidia from a hygromycin-resistant *F. oxysporum* strain that does not infect tomato (*Fo*-47) and double-drug-resistant strains thus obtained were able to cause wilt disease on tomato (Ma et al. 2010) (Fig. 7.2a). All the double-drug-resistant strains (*Fo*-47+) had a karyotype similar to the *Fo*-47 parent except for the presence of one or two small chromosomes from *Fol*-007 (Fig. 7.2b). All the pathogenic *Fo*-47+ strains were found to carry the chromosome 14 from *Fol*-007 (Fig. 7.2c), and two of the pathogenic ones, which showed highest level of virulence, also received another chromosome corresponding to the smallest chromosome of *Fol*-007. This smallest chromosome was positive for one marker associated with a 1.3-Mb region from chromosome 3 and another marker for a 1.0-Mb region on chromosome 6. Both of these regions correspond to large duplicated lineage-specific regions of the *F. oxysporum* f. sp. *lycopersici* genome. None of the conserved chromosomes were transferred. Thus, acquisition of the *Fol*-007 chromosome 14 by *Fo*-47 rendered the non-pathogenic *Fo*-47 capable of infecting plants (Ma et al. 2010). Interestingly, no double-drug resistant strain containing a tagged chromosome of *Fo*-47 in *Fol*-007 background could be obtained even when a randomly generated Zeocin-resistant *Fol*-007 strain was used. This preferential transfer may be determined by factors like size, composition and stability of the mobile chromosome. Although information is lacking on how frequently they occur under natural conditions, existence of such a transfer process threatens the practice of using non-pathogenic *Fusaria* as biological control, many of which are used on a commercial scale.

Transfer of supernumerary chromosomes has also been experimentally demonstrated in *Colletotrichum gloeosporioides* and *Alternaria alternata*. In *Colletotrichum gloeosporioides*, two distinct pathotypes, types A and B, exist that are morphologically identical in culture but are asexual, vegetatively incompatible and genetically distinct (He et al. 1998). A 2-Mb supernumerary chromosome is present in pathotype A and some, but not all, field isolates of pathotype B. Cocultivation of a pathotype A transformant carrying hygromycin resistance marker in the 2-Mb supernumerary chromosome with phleomycin resistance pathotype B resulted in double-drug-resistant transformants carrying the entire 2-Mb supernumerary chromosome from pathotype A (He et al. 1998). Like chromosome 14 of *F. oxysporum* f. sp. *lycopersici*, double-drug resistance could only be obtained when the hygromycin resistance marker was present in the 2-Mb supernumerary chromosome.

Alternaria alternata is another asexual filamentous fungus where both pathogenic and non-pathogenic types occur. A small conditionally dispensable chromosome of varying size carrying genes for *Alternaria* toxin biosynthesis is present in the pathogenic isolates of this fungus (Akagi et al. 2009; Akamatsu et al. 1999; Hatta et al. 2002; Johnson et al. 2001). Spontaneous or induced loss of this extra chromosome in tomato and apple pathotypes of *A. alternata* resulted in loss of pathogenicity (Johnson et al. 2001; Akagi et al. 2009). Protoplast fusion of tomato and strawberry pathotypes produced a fusant that could infect both tomato and strawberry. This fusant produced both tomato and strawberry pathotype-specific toxins, and conditionally dispensable chromosomes derived from both the parental pathotypes were detected in the hybrid. The hybrid's genetic make-up was that of the strawberry pathotype except for the additional pathogenicity chromosome from the tomato pathotype.

While this demonstrates that conditionally dispensable chromosomes can be transferred and stably maintained in a new genome, how this occurs remains a question. Although formation of bridges though pili or pili-like structures is not reported in fungi, fusion between fungal cells can occur through conidial, germ tube or hyphal anastomosis (Read and Roca 2006; Roca et al. 2006). While conidial anastomosis and germ tube fusions appear to be different processes, similar regulatory processes are involved in conidial and hyphal fusions. In *F. oxysporum*, fusion between germinating conidia and vegetative hypha frequently occurs at early stages of plant infection (Mesterhazy 1973; Rosales and Di Pietro 2008; Ruiz-Roldan et al. 2010), and although not essential for plant infection, such fusion appears to contribute towards effective adhesion and colonisation of the root surface (Rosales and Di Pietro 2008). Hyphal fusion is achieved through directed growth of fusing hyphae towards each other and formation of anastomosis tubes (Ruiz-Roldan et al. 2010). Once the bridge is formed, one of the nuclei undergoes division and the daughter nucleus moves into the recipient compartment through the anastomosis tube. After that, the resident nucleus is degraded (Ruiz-Roldan et al. 2010). Similar movement of nucleus has been observed in conidial anastomosis in *Colletotrichum* and *N. crassa* (Roca et al. 2003, 2005). Such heterokaryosis can result in mitotic crossing over and haploidisation leading to parasexual recombination and thus contribute to genetic variation, including horizontal gene or chromosome transfer in fungi lacking sexual production. Anastomosis between individuals is controlled by *Het* or *Vic* loci (heterokaryon or vegetative incompatibility) and most frequently observed between genetically identical individuals (self-anastomosis; Glass et al. 2000). Although heterokaryons formed between non-identical genotypes of different *Het* group are followed by cell death or severe growth impairment, non-self-anastomosis (both intra- and interspecies) has been reported to occur (Qu et al. 2008; Roca et al. 2004; Toda and Hyakumachi 2006), and slow-growing heterokaryons formed have been suggested as an intermediate form through which horizontal chromosome transfer can take place (Manners and He 2011). In a recent study it was observed that fused conidia from incompatible strains of *C. lindemuthianum* could escape incompatibility triggered cell death for at least 30 min (Ishikawa et al. 2012). Following anastomosis, heterokaryon

formation and nuclear fusion or transfer of nuclear proteins were noted which at times resulted in uninucleate heterokaryons. Colonies formed from such uninucleate heterokaryons were resistant to both the antibiotics to which parental strains were individually resistant, and their race specificities were altered as well (Ishikawa et al. 2012). This strongly suggests that conidial anastomosis in *C. lindemuthianum* can survive, at least under the experimental conditions; incompatibility-related cell death and parasexual recombination can occur in such cases leading to new genetic variability. Such a scenario could explain horizontal gene and/or chromosome transfers between genetically distant lineages of *F. oxysporum*, emergence of new pathogenic lineages and their apparent polyphyletic origins. Loading one mobile chromosome with pathogenicity genes would be of great ecological advantage to the fungus for such one-step conversion of a benign strain into a pathogenic one on a new host plant. What is the frequency of such transfer in nature, what are the proteins involved in such flow of genetic information, how such a process would be controlled and whether transposable elements and repeats present in supernumerary chromosomes plays a role in horizontal transfers are questions that need answers.

7.11 Outlook

In recent years, genome sequencing and analysis of plant pathogenic *Fusarium* spp. has shed new lights into both molecular mechanisms and evolution of pathogenicity. While major progress has been made in the discovery of small effector proteins, lots remain to be determined about their role in disease incidence and their mode of action. It is expected that these findings will help devise novel strategies for management of the menace called Fusaria.

Another finding of immense practical significance is that most of the host-specific virulence genes are present on lineage-specific chromosome(s) that are devoid of house-keeping genes and that these chromosomes can be horizontally transferred to non-pathogenic strains rendering these pathogenic. Horizontal chromosome transfer can explain independent evolution of host-specific virulence within a forma specialis where members are grouped together based on their virulence on a host but are genetically distant. While the universality of this phenomenon needs to be established involving more members of this group, a possibility exists that many non-pathogenic Fusaria used as biocontrol agents for management of pathogenic Fusaria (competitive exclusion) can acquire novel virulence through horizontal transfer of "pathogenicity chromosomes" which might pose a serious problem in future. Till questions are answered regarding mechanism, natural occurrence and role of horizontal chromosome transfer in evolution of *Fusarium*, use of non-pathogenic strains as a mean to control *Fusarium* diseases should be dealt with caution.

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Chapter 8
Genomics and Spectroscopy Provide Novel Insights into the Mechanisms of Litter Decomposition and Nitrogen Assimilation by Ectomycorrhizal Fungi

Anders Tunlid, Francois Rineau, Mark Smits, Firoz Shah, Cesar Nicolas, Tomas Johansson, Per Persson, and Francis Martin

8.1 Introduction

A large part of the nitrogen (N) in forest soils is present in organic forms including proteins but also other compounds like amino sugars and heterocyclic N molecules (Nannipieri and Eldor 2009). Several studies have demonstrated that forest trees have the capacity to acquire organic N in the form of amino acids. However, free amino acids generally account for only a small fraction of the organic N pool, and the ecological significance of this uptake is a matter of discussion (Näsholm et al. 2009). Moreover, organic N compounds in soils are found in association with polyphenols, polysaccharides, and other degradation products of plant and microbial polymers (Piccolo 2001). To get access to the organic N embedded in such complexes of soil organic matter (SOM), at least part of the shielding compounds, including polyphenols, needs to be degraded. For these reasons, it is generally thought that forest trees are dependent on the depolymerizing activity of microorganisms to access the organic N fraction (Schimel and Bennett 2004; Read

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*,
Soil Biology 36, DOI 10.1007/978-3-642-39339-6_8,
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and Perez-Moreno 2004). Frank (1894) proposed already at the end of the nineteenth century that fungal symbionts, i.e., mycorrhizal fungi, might be one of the key organisms performing this activity. However, the "organic nitrogen theory" of mycorrhizal fungi was largely ignored until the mid-1980s (Trappe 2005; Read and Perez-Moreno 2003). As a result, the view has emerged that soils contain two distinct functional groups of fungi: the decomposers, which are involved in the decomposition of complex organic material and release of their nutrient content, and the fungal mutualists that absorb the mineral nutrients released by the decomposition process.

In boreal forests saprotrophic fungi are primarily found in the surface litter layers, while mycorrhizal fungi are dominating in underlying layers containing more decomposed litter and humic-rich SOM (Lindahl et al. 2007). Humic substances originate from the microbial decay of leaf litter, root litter, and root exudates. The main constituent of the plant litter is lignocellulose. Lignin, one of the most abundant components of lignocellulose, is a recalcitrant aromatic polymer consisting of phenylpropane units joined by C–C and ether linkages (Ralph et al. 2004). During the initial stages of SOM decay, the lignin polymers are chemically modified, and C and nutrients become available for saprotrophic fungi and bacteria, thereby promoting further degradation. The undecayed portion of the plant litter remains in the soil together with microbially derived compounds and residues. This mixture, operationally defined as humic substances, consists of relatively small molecules that associate with each other in supramolecular aggregates. These aggregates are stabilized by hydrophobic interactions and hydrogen (H) bonding, and their properties are determined by all of the constituents and their associations (Sutton and Sposito 2005) (Fig. 8.1).

Lignin decay mechanisms have been studied almost exclusively in the context of wood decomposition. Two main types of mechanisms have been characterized in detail: decomposition by white-rot fungi and by brown-rot fungi (Hatakka and Hammel 2010). The white-rot fungi degrade all components of lignocellulose including cellulose, hemicelluloses, and lignin. Lignin degradation in these fungi depends on the concerted action of dedicated peroxidases and hydrolytic enzymes that include various cellulases and hemicellulases. The brown-rot fungi have developed a different decay strategy being able to degrade most of the cellulose and hemicelluloses, leaving the lignin polymer essentially undigested albeit chemically modified. The brown-rot fungi express a distinct set of hemicellulases, endoglucanases, and oxidases during the degradation of lignocellulosic material but lack ligninolytic peroxidases, as well as many of the exocellulases present in white-rot fungi. Of major importance for the disruption of the lignin barrier of brown-rot fungi is the production of free radicals by a Fenton mechanism (Martinez et al. 2009; Eastwood et al. 2011). During this reaction, Fe^{2+} reacts with H_2O_2 to produce hydroxyl radicals ($OH^{\cdot}$) that can unspecifically oxidize both lignin and cellulose molecules (Fig. 8.1).

In contrast to the decay of lignin in wood, little is known about the decomposition of lignin residues and other recalcitrant aromatic compounds in humic-rich SOM. Recently, it was shown that the genome of the humicolous fungus *Agaricus*

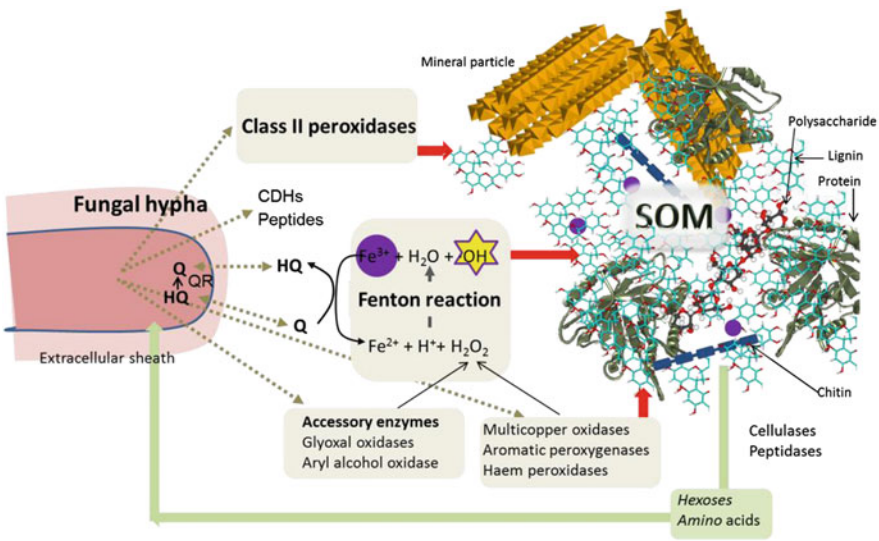


Fig. 8.1 A schema of the complex interactions between fungal hyphae and soil components, which ultimately determine the extent and rate of soil organic matter (SOM) degradation (see main text for explanations)

bisporus is rather similar to those of wood-decaying fungi (Morin et al. 2012). The genome of this fungus encodes a full repertoire of polysaccharide-degrading enzymes similar to that of wood decayers. However, a striking expansion of gene families encoding heme-thiolate peroxidases and β-etherases is distinctive from the Agaricomycotina wood decayers and suggests abilities to broadly attack lignin residues in humic-rich environments (Morin et al. 2012).

The interfaces between microorganisms and soil constituents, including SOM and soil mineral particles, are the zone in which the oxidative SOM degradation reactions occur (Fig. 8.1). These complex interfaces constitute less than 1 % of the soil volume and are distributed heterogeneously in small-scale habitats, connected by water-saturated or water-unsaturated pore space. The biological, chemical, and physical heterogeneity of these microhabitats is likely to have a large effect on the turnover of SOM (Schmidt et al. 2011). At the molecular level, this turnover is controlled by the properties of the fungal hyphae–SOM–mineral assemblies.

The interactions between the ensemble of biologically produced molecules required for oxidative degradation and the components of these assemblies need to be uncovered to understand C decomposition at the molecular level. We consider that this can be accomplished only by unifying experimental information about the gene expression of the fungi with information about the molecular structure and properties of the SOM (Fig. 8.2). The growing wealth of genome sequences of fungi and the application and development of novel spectroscopic methods have open up new possibilities for such research. This review covers some recent studies using such approaches and the insight that they provide regarding mechanisms for litter

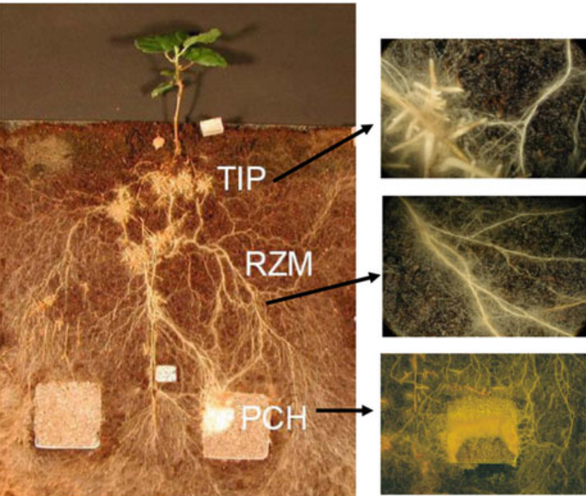


Fig. 8.2 The extrametrical mycelium developed from an ectomycorrhizal fungus. A birch (*Betula pendula*) seedling colonized by the ectomycorrhizal fungus *Paxillus involutus*. The fungus colonizes the plant's root tips (TIP). Supported by plant C, the fungal mycelia grow extensively and fuse to form thick rhizomorphs (RZM). When the mycelia encounter a patch (PCH) with organic material, the material is degraded, and the assimilated nutrients are transferred to the plant. Reprinted from Wright et al. (2005) with permission

decomposition and N assimilation by ectomycorrhizal (ECM) fungi. However, we will first give a short background to the ecology of litter decomposition by ECM fungi highlighting areas of uncertainty.

8.2 Ectomycorrhizal Fungi as Decomposers

8.2.1 The Extramatrical Mycelium: The Interface Between ECM and Soil

Ectomycorrhizae (ECM) is the dominant type of mycorrhiza found in association with tree roots in a boreal forest (Smith and Read 1997). Development of the ECM tissue proceeds through the differentiation of a fungal mantle surrounding the plant root, inwardly directed hyphae which form the Hartig net around root cortical cells and externally directed hyphae forming the extramatrical mycelium in the soil (Fig. 8.2). Supported by carbon (C) from the host plant, the extramatrical mycelium can grow extensively within the soil and efficiently prospect for nutrients, often at considerable distances from the plant root system (Read and Perez-Moreno 2003). The mycelial system is regarded as indeterminate, structurally and physiologically heterogeneous network that interconnects multiple plant root systems (Cairney and Burke 1996). The development and differentiation of the extramatrical mycelia

differ between different taxa of ECM fungi, and it has been proposed that the morphology of the mycelia can be used as a trait to classify ECM into various ecological groups (Agerer 2001). Such exploration types explore the surrounding soils by extramatrical mycelia which are either concentrated in vicinity of the mycorrhizal mantle or formed as far-reaching strands (i.e., rhizomorphs) (cf. Fig. 8.2). When encountering a patch of nutrients, the hyphae can proliferate extensively. Hydrolytic and oxidative enzymes are secreted, and nutrients are released and assimilated by the mycelium and transported to the host plant. Once the organic material is fully colonized by the fungus and the available nutrients withdrawn, the amount of plant C allocated to the mycelium in the patch rapidly decreases, and the mycelium dies (Leake et al. 2001). Residues of the ECM fungal mycelium can persist in soil for long time and may constitute a large fraction of SOM (Clemmensen et al. 2013).

Direct evidences that ECM fungi could assimilate organic N from complex organic matter and transfer the N to the host plant emerged from a number of seminal experiments performed by David Read and colleagues using soil microcosms (Abuzinadah et al. 1986; Read and Perez-Moreno 2003). By using soil microcosms, it could be demonstrated (Abuzinadah et al. 1986) that mycorrhizal plants of *Pinus contorta* associated with the ECM fungi *Paxillus involutus*, *Suillus bovinus*, and *Rhizopogon roseolus* could grow on a substrate supplemented with protein as the sole N source. Since then, numerous studies have shown that ECM fungi can assimilate organic N from proteins and other complex natural organic substrates like litter material, pollen grains, and chitin (that is the main component of necromass of fungal mycelia and soil mesofauna) (Read and Perez-Moreno 2004). In these experiments the N sources were exploited and a significant portion of N was delivered to the host plant (Read and Perez-Moreno 2004). More recently, field studies using isotope analyses also provide evidences that ECM fungi mobilize N during litter decomposition in boreal forest soils (Lindahl et al. 2007).

8.2.2 Nutrient-Mobilizing Enzymes

Assimilation of N from organic sources requires the secretion of a range of different enzymes. It has been reported that ECM fungi can indeed express extracellular enzymes that can degrade proteins and, at least to some extent, other organic compounds in plant-litter material (such as cellulose, hemicellulose, and polyphenols) in which N is embedded (Zhu et al. 1990; Norkrans 1950; Haselwandter et al. 1990; Trojanowski et al. 1984). Moreover studies of soil microcosms have shown that the assimilation of N from patches of plant-litter material by the ECM fungus *P. involutus* is associated with an enhanced protease and polyphenol oxidase activity (Bending and Read 1995).

There have been numerous attempts to measure the activities of nutrient-mobilizing enzymes secreted by ECM in the field. Microplate enzymatic tests

have been developed to measure secreted enzyme activities by freshly harvested ECM root tips. These assays are using commercially available substrates for peptidase, hydrolytic and oxidative enzymes involved in the decomposition of lignocellulose, chitin, and phosphorous containing organic compounds (Courty et al. 2005). These cultivation-independent methods have been used in field studies examining the temporal and spatial dynamics of enzyme activities involved in nutrient cycling and how they are influenced by disturbance (Pritsch and Garbaye 2011). One intriguing result from these studies is that the enzyme activities were elevated at some periods when the level of photosynthate was low (Courty et al. 2007; Cullings et al. 2008). This observation suggests that ECM fungi may live as facultative saprotrophs, i.e., they can degrade and metabolize SOM as an alternative C source when the energy supply from the host plants is low. Hence, it has been proposed that the saprotrophic activity of ECM fungi might represent a significant pathway of C loss in forest ecosystems (Talbot et al. 2008).

8.2.3 The Mechanism of Litter Decomposition Is Unclear

However, the efficiency by which ECM fungi can degrade and metabolize SOM is not clear, and the evidences that they can act as facultative saprotrophs have been questioned. As compared with saprotrophic fungi, ECM genome sequence information show a reduced set of genes encoding plant cell wall-degrading enzymes (Martin et al. 2008; Nagendran et al. 2009), which suggests that ECM fungi have limited capacity to degrade plant-litter material. Furthermore, there are several reports showing that ECM fungi have—particularly in comparison with saprophytic fungi—only a limited enzymatic ability to degrade and mobilize N from protein–polyphenol complexes (Bending and Read 1996; Wu et al. 2003). The conclusions drawn from field studies using enzyme activity profiling have also been questioned based on the fact that the assays are unspecific and therefore may overestimate the decomposing activities of ECM fungi (Baldrian 2009). Moreover, it has been argued that the ECM fungi are mostly found in humic soil layer (Lindahl et al. 2007), which is composed only of low-energy-content molecules that cannot support an extensive saprotrophic growth of ECM fungi (Baldrian 2009).

8.3 Conversion of Organic Matter by ECM Fungi

8.3.1 Probing Chemical Modifications of SOM Using Spectroscopy

To examine the mechanisms by which ECM fungi degrade complex organic material extracted from plant-litter material, the fungus *Paxillus involutus* was grown in an axenic model system that allowed careful control of growth conditions

and precise sampling of the substrate for spectroscopic analyses and harvesting of mycelium for transcriptome profiling (Rineau et al. 2012). *P. involutus* (Batsch) Fr. (Basidiomycetes, Boletales) is widely distributed in the Northern Hemisphere and is one of the best-studied ECM fungi, especially with respect to the physiology related to the uptake and transformation of nutrients (Wallander and Söderström 1999). The SOM constituents of organic material extracted from forest litter and from maize compost material. Low-molecular-weight material such as inorganic N sources was removed from the extracts by ultrafiltration, and the fungus was starved for N before the SOM extracts were added.

A range of spectroscopic techniques was used to characterize the conversion of the major components of the SOM by *P. involutus*. Fourier transform infrared (FTIR) spectroscopy provides a chemical fingerprint of the functional group composition (e.g., carbonyls, carboxyls, hydroxyls, phenols, and phenyls) as well as information on the alteration of these groups as a result of degradation. Synchronous fluorescence spectroscopy was used for a more detailed characterization of the aromatic molecules. This technique allows for measurement of the degree of condensation and substitution of humic substances (Senesi et al. 1991). Finally, a quantitative in-depth analysis of the composition of the SOM was conducted by pyrolysis gas chromatography (Py-GC/MS). This technique also allowed the measurement of the degree of oxidation of the lignin building blocks. The results obtained with those three techniques demonstrated that, while *P. involutus* assimilated half of the organic N in the SOM, the polyphenols and polysaccharides were at least partially degraded. Of particular interest were the chemical modifications of the lignin residues as detected by Py-GC/MS (Fig. 8.3). They included the removal and oxidation of side chains, which are modifications that have also been observed during the degradation of lignin through Fenton reactions by brown-rot fungi (Martinez et al. 2011; Yelle et al. 2011).

8.3.2 Production of Iron-Reducing Compounds

A key requirement for the Fenton mechanism is a system for reduction of Fe^{3+} to Fe^{2+}, which might be accomplished by extracellular fungal metabolites or reductive enzymes (Hatakka and Hammel 2010). The iron-reducing activity was indeed significantly increased during decomposition of the organic matter extracts by *P. involutus* (Rineau et al. 2012), suggesting that iron-reducing compound(s) was secreted during organic matter degradation. The chemical structures and the role of secondary metabolites in iron redox cycling have been studied in the brown-rot bolete *Serpula lacrymans* (Eastwood et al. 2011). It remains to be determined whether *P. involutus* produces similar metabolites during the decomposition of plant-litter material.

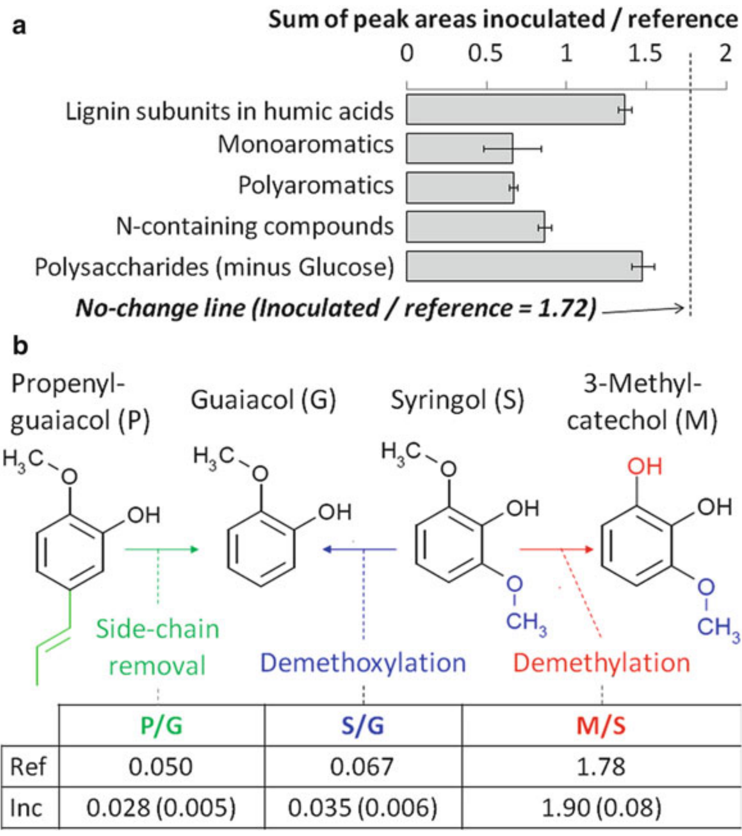
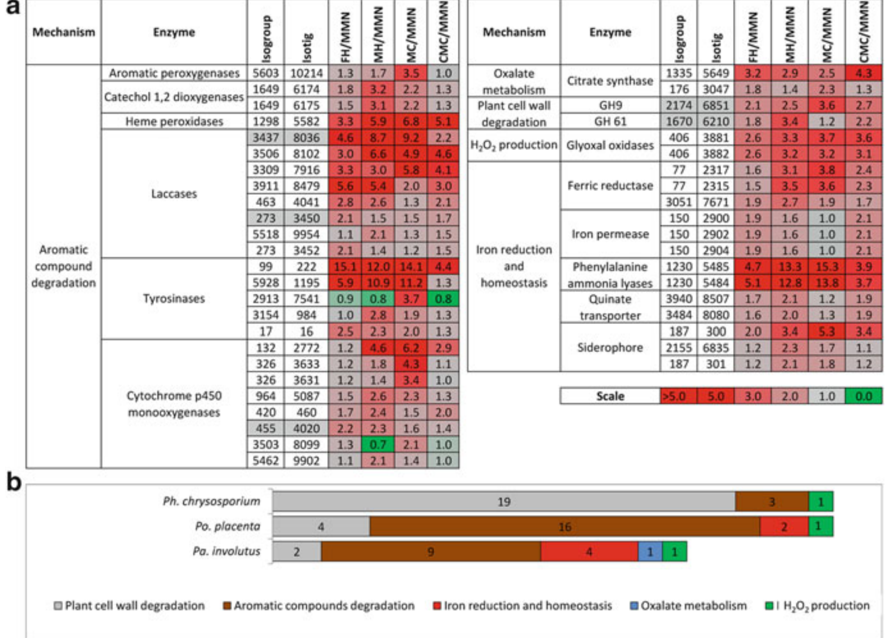


Fig. 8.3 Degradation of organic material extracted from forest litter by *P. involutus*. The material was analyzed using pyrolysis GC/MS after 7 days of incubation (Inoculated) and before incubation (Reference). (**a**) Relative amounts of the major groups of organic compounds. A ratio below the "no change line" indicates that this particular class of pyrolysis products was depleted in the Inoculated as compared to the Reference samples. "Lignin" does not refer to genuine plant lignin but rather parts of the lignin molecule that are present in the humic acids as residuals of the degradation process. (**b**) Chemical modification of lignin residuals subunits. Numbers indicate the relative peak area of the different lignin subunits in the reference sample against the average relative peak area in the incubated samples ($N = 5$, error bars denote standard error). Reprinted from Rineau et al. (2012) with permission

8.3.3 Trancriptome Profiling

The involvement of a Fenton-based biodegradation system during the decomposition of the SOM extracts was confirmed by analyzing the transcriptional response in *P. involutus* using DNA microarrays. The array contained probes for 12,214 transcripts (isotigs) that were obtained by sequencing the transcriptome of *P. involutus* when grown on the SOM extracts (Rineau et al. 2012). Based on manual annotations, we identified 269 transcripts encoding enzymes known to be



a

Mechanism	Enzyme	Isogroup	Isotig	FH/MMN	MH/MMN	MC/MMN	CMC/MMN
Aromatic compound degradation	Aromatic peroxygenases	5603	10214	1.3	1.7	3.5	1.0
	Catechol 1,2 dioxygenases	1649	6174	1.8	3.2	2.2	1.3
		1649	6175	1.5	3.1	2.2	1.3
	Heme peroxidases	1298	5582	3.3	5.9	6.8	5.1
	Laccases	3437	8036	4.6	8.7	9.2	2.2
		3506	8102	3.0	6.6	4.9	4.6
		3309	7916	3.3	3.0	5.8	4.1
		3911	8479	5.6	5.4	2.0	3.0
		463	4041	2.8	2.6	1.3	2.1
		273	3450	2.1	1.5	1.5	1.7
		5518	9954	1.1	2.1	1.3	1.5
		273	3452	2.1	1.4	1.2	1.5
	Tyrosinases	99	222	15.1	12.0	14.1	4.4
		5928	1195	5.9	10.9	11.2	1.3
		2913	7541	0.9	0.8	3.7	0.8
		3154	984	1.0	2.8	1.9	1.3
		17	16	2.5	2.3	2.0	1.3
	Cytochrome p450 monooxygenases	132	2772	1.2	4.6	6.2	2.9
		326	3633	1.2	1.8	4.3	1.1
		326	3631	1.2	1.4	3.4	1.0
		964	5087	1.5	2.6	2.3	1.3
		420	460	1.7	2.4	1.5	2.0
		455	4020	2.2	2.3	1.6	1.4
		3503	8099	1.3	0.7	2.1	1.0
		5462	9902	1.1	2.1	1.4	1.0

Mechanism	Enzyme	Isogroup	Isotig	FH/MMN	MH/MMN	MC/MMN	CMC/MMN
Oxalate metabolism	Citrate synthase	1335	5649	3.2	2.9	2.5	4.3
		176	3047	1.8	1.4	2.3	1.3
Plant cell wall degradation	GH9	2174	6851	2.1	2.5	3.6	2.7
	GH 61	1670	6210	1.8	3.4	1.2	2.2
H_2O_2 production	Glyoxal oxidases	406	3881	2.6	3.3	3.7	3.6
		406	3882	2.6	3.2	3.2	3.1
Iron reduction and homeostasis	Ferric reductase	77	2317	1.6	3.1	3.8	2.4
		77	2315	1.5	3.5	3.6	2.3
		3051	7671	1.9	2.7	1.9	1.7
	Iron permease	150	2900	1.9	1.6	1.0	2.1
		150	2902	1.9	1.6	1.0	2.1
		150	2904	1.9	1.6	1.0	2.1
	Phenylalanine ammonia lyases	1230	5485	4.7	13.3	15.3	3.9
		1230	5484	5.1	12.8	13.8	3.7
	Quinate transporter	3940	8507	1.7	2.1	1.2	1.9
		3484	8080	1.6	2.0	1.3	1.9
	Siderophore	187	300	2.0	3.4	5.3	3.4
		2155	6835	1.2	2.3	1.7	1.1
		187	301	1.2	2.1	1.8	1.2

Scale	>5.0	5.0	3.0	2.0	1.0	0.0

Fig. 8.4 Regulation of genes potentially involved in organic matter degradation by *P. involutus*. (**a**) Expression profile of 44 genes that were manually annotated as potentially involved in organic matter degradation and were upregulated more than twice (false discovery rate $q < 0.01$) in at least one of pairwise comparisons in media containing extracts of complex organic material versus mineral nutrient medium (MMN). The data presented are average ratio of expression ($N = 3$). Four different types of organic substrates were used: forest litter extracted with hot water (FH), maize compost extracted with hot water (MH), maize compost extracted with cold water (MC), and carboxymethyl cellulose (CMC). Isotigs and isogroups refer to transcripts and genes, respectively. In *gray boxes* are four isotigs that were also identified in a (TAST) screening for secreted proteins. (**b**) Comparison of the transcriptional response of *P. placenta*, *P. chrysosporium*, and *P. involutus* when growing on a cellulose medium as compared to a medium containing glucose as the carbon source. The number of genes that were upregulated at least twofold (in average of three replicates) and those with annotations consistent with a potential role in organic matter degradation are shown. Reprinted from Rineau et al. (2012) with permission

involved in the degradation of organic matter in white-rot and brown-rot fungi. In total, 44 of these transcripts were significantly upregulated during the conversion of organic material. Notably, the set of genes was similar to those expressed by the brown-rot fungus *Postia placenta* when grown on cellulose medium (Fig. 8.4). When in contact with organic matter, *P. involutus* overexpressed a number of transcripts of oxidases like laccases, catechol dioxygenase, heme peroxidase, tyrosinases, and cytochrome p450 monooxygenases that have also been shown to be produced by the brown-rot fungus *P. placenta* when grown on wood or cellulose media (Martinez et al. 2009; Vanden Wymelenberg et al. 2010). No transcripts encoding class II peroxidases (Mn or lignin peroxidases) that are signatures for a white-rot mechanism were detected in the transcriptome of *P. involutus*.

However, considering the expression of genes encoding carbohydrate-active enzymes (CAZymes) involved in the degradation of the plant cell wall, there were large differences between the symbiotic *P. involutus* and saprophytic *P. placenta*. Although, none of them expressed genes coding for enzymes of the canonical crystalline cellulose decomposition system (Lynd et al. 2002), the transcriptional profile of *P. placenta* employs an array of CAZymes like endoglucanases, β-glucosidases, and hemicellulase when it is grown on cellulose or aspen (Martinez et al. 2009; Vanden Wymelenberg et al. 2010). Except for endoglucanase (GH9), no glycosyl hydrolases were induced in *P. involutus* during growth on plant litter or cellulose. In addition to the GH9, a member of the GH61 family was significantly upregulated in *P. involutus*. GH61 is the most abundant CAZyme family acting on the plant cell wall in the genome of *L. bicolor* (Martin et al. 2008). Recently, it has been reported that GH61 can depolymerize cellulose oxidatively in cooperation with cellobiose dehydrogenase or low-molecular-weight reducing agents (Langston et al. 2011; Quinlan et al. 2011). Hence, GH61 could be an important component of the radical-based cellulose-degrading mechanism of ECM fungi.

The lack of transcription of extracellular CAZymes needed for metabolizing C released by the Fenton reaction could be expected in *P. involutus* considering the fact that many of these enzymes are lacking in the genome of *P. involutus* (A. Tunlid, F. Martin et al. unpublished data). This observation implies that the decomposing mechanism has been tuned for assimilating organic N rather than C from the SOM and that the ability to assimilate the released C has been lost as an adaptation to growth on the host photosynthate (Rineau et al. 2012).

8.3.4 Ecological Implications of a Trimmed Saprophytic Activity

The C and nutrients released during the radical-based decomposition of SOM by fungi may become available for further degradation and assimilation by saprotrophic microorganisms. Several studies have shown that the mycelia of ECM fungi are surrounded by distinct communities of saprophytic bacteria and fungi (De Boer et al. 2005; Izumi and Finlay 2011). Most likely, some of these microorganisms are commensals that grow on the C resources that become available during the radical-based degradation. Other microorganisms may be competitors that strive for the same nutrient resources as the ECM fungi. Thus, it can be expected that ECM fungi have evolved mechanisms that could control the activity of saprophytic microorganisms. The first evidence for such a mechanism was reported already in 1971, when it was demonstrated that the presence of mycelium of ECM fungi suppressed litter decomposition (Gadgil and Gadgil 1971). More recently, studies in soil microcosms have shown that the ECM mycelium of *P. involutus* can reduce the activity of saprophytic bacteria (Olsson

et al. 1996). The mechanism of the "Gadgil effect" is not clear; proposed explanations are the production of toxic metabolites and the removal of nutrients or water by the ECM mycelium (Bending 2003).

8.4 Regulation of the Decomposing Activities in ECM Fungi by Carbon and Nitrogen

8.4.1 Carbon Availability Triggers SOM Decomposition

The finding that *P. involutus* does not express the hydrolases needed for metabolizing the released C fragments suggests that the decomposing activity is dependent on sugars from the host plant. Assuming that glucose is the main form of plant C supplied to the ECM fungus (Nehls et al. 2010), we have recently examined the decomposition of litter material regulated by glucose (Rineau et al. 2013). Spectroscopic analyses revealed that degradation of SOM did only occur when glucose was added to the extracts. Hence, glucose triggered the decomposition of the litter material. Concomitantly, the expression of genes encoding enzymes involved in oxidative degradation of polysaccharides and polyphenols (i.e., Fenton chemistry) was upregulated. Addition of glucose also stimulated the assimilation of N and the expression of genes encoding enzymes and transporters of all the major steps of organic N assimilation of ECM fungi (Rineau et al. 2013). This involved the degradation of proteins, uptake of released amino acids and peptides, and internal transformation of amino acids. Taken together, data supports the hypothesis that the SOM-degrading activity and assimilation of N by *P. involutus* is dependent on sugars from the host plant.

The physiological mechanism providing the stimulating effects of glucose is not known. Adding glucose to the extracts of SOM significantly stimulates the expression of metabolic pathways involved in energy generation (glycolysis, pyruvate metabolism, and the TCA cycle). Transcripts encoding enzymes mediating the oxidative parts of the pentose phosphate pathway are also upregulated. Thus, apart from generating energy, the added glucose most likely stimulates the production of reducing power (NADPH) needed for biosynthetic reactions. Another possibility is that the added glucose triggers the activity of extracellular enzymes involved in the oxidation of the SOM. Such mechanisms have been proposed to explain the so-called priming effects, i.e., the stimulation of SOM turnover caused by the addition of easily available organic C or N sources (Bengtson et al. 2012).

The above findings suggest that the regulation of the plant cell wall-decomposing machinery may differ significantly between saprophytic and ECM fungi. In saprophytic fungi, the expression of plant cell wall-degrading enzymes including cellulases, hemicellulases, ligninases, and pectinases is commonly repressed in the presence of glucose (Aro et al. 2005). Furthermore, several studies of the brown-rot fungus *P. placenta* have shown that the oxidative degradation of

cellulose and wood substrates can occur without adding glucose to the medium (Martinez et al. 2009; Vanden Wymelenberg et al. 2010). Accordingly, both a reduction in gene content of plant cell wall-degrading enzymes and mutations affecting the transcriptional regulation have contributed to the symbiotic adaptations of the decomposing mechanism in ECM fungi in the Boletales.

8.4.2 Ammonium Has Relatively Minor Effects on the Decomposing Activities

The fact that the decomposition of SOM by *P. involutus* is linked to the degradation of organic N suggests that the degradation activity should be controlled by environmental factors known to regulate the activity of enzymes involved in the breakdown of soil organic N sources such as proteins. In many filamentous fungi including the ECM fungus *Hebeloma crustuliniforme*, the expression of proteolytic activity is repressed in the presence of the favorable (or primary) N source like ammonium (Marzluf 1996; Zhu et al. 1994). Moreover, studies in saprophytic fungi have shown that N availability may also affect the expression levels of lignocellulolytic enzymes including cellulases, peroxidases, and laccases (Aro et al. 2005; Fenn and Kirk 1981; Chen et al. 2003; Edwards et al. 2011). However, the addition of ammonium—which is the most abundant form of inorganic N in boreal forest soils—had relatively minor effects on both the transcription and the decomposition of litter material by *P. involutus* and only occurring when glucose was present (Rineau et al. 2013).

8.4.3 Interactions Between C and N

ECM fungi assimilate nutrients including N from soil and exchange them for photosynthetically fixed C. The mechanism that regulates this exchange is not known, but it has been proposed that the plant host reduces the C supply to the fungus if it fails to supply adequate amount of nutrients (Nehls et al. 2007). Recent studies on the arbuscular mycorrhizal (AM) symbiosis have demonstrated that the symbiosis is stabilized by reciprocal rewarding of nutrients and C resources according with the nutritional benefit provided by the other partner (Kiers et al. 2011). Experiments using AM mycorrhizal root organ cultures have also shown that the C availability of the plant triggers the uptake and transport of N uptake by the AM fungus (Fellbaum et al. 2012). The finding that *P. involutus* does not degrade litter material without glucose suggests that the C flux from the host plant can control the assimilation of organic N in ECM fungi. Studies of ECM fungi in association with a host plant will be needed to verify this hypothesis.

Moreover, regulation of the decomposing activities of ECM fungi by the plant photosynthate needs to be considered for the understanding of environmental factors that control the C cycles in forest ecosystem. Talbot et al. (2008) proposed three hypotheses for mechanisms by which mycorrhizal fungi act as decomposers. The first hypothesis ("Plan B") is that mycorrhizal fungi metabolize significant quantities of SOM when the supplies of plant photosynthate are low. The second hypothesis ("Coincidental Decomposer" hypothesis) is that mycorrhizal fungi decompose soil C as a consequence of mining SOM for organic nutrients. The third hypothesis ("Priming Effects" hypothesis) is that mycorrhizal fungi decompose SOM when allocation of plant C to mycorrhizal roots is high. Data from the study by Rineau et al. (2013) provides support for both the "Priming Effect" and "Coincidental Decomposer" hypotheses, but not for the "Plan B" hypothesis.

8.5 Molecular Components of the Protein Degradation Pathways in ECM Fungi

Proteins contribute to major part of the organic N in forest soils (Nannipieri and Eldor 2009). The assimilation of N from proteins involves several steps including the degradation of organic N polymers, assimilation of released mono- and oligomers, internal metabolism, and transfer to the host plant (Chalot and Brun 1998; Talbot and Treseder 2010). Proteases are key enzymes involved in the extracellular degradation of proteins by fungi, and experiments have shown abilities of *P. involutus* to capture N from plant-litter material associated with increased protease activities in colonized material (Bending and Read 1995). Furthermore, studies in pure culture systems using protein as a sole N source have shown that abilities to produce extracellular proteases are common among ECM fungi (Ramstedt and Söderhäll 1983; Zhu et al. 1990; Nehls et al. 2001; Nygren et al. 2007). The proteolytic activity is typically expressed at an acidic pH (<5.0) and is mainly inhibited by compounds active against aspartic proteases. Characterizations of the extracellular proteolytic activity in *Hebeloma crustuliniforme* and *Amanita muscaria* have shown that this is due to aspartic proteases (Zhu et al. 1990; Nehls et al. 2001). More recently, analysis of the genome and transcriptomes of *L. bicolor* reveals that ECM fungi can express a large number of proteases and peptidases, not only including aspartic proteases but also members of the serine, metallo, and cysteine classes of peptidases (Martin et al. 2008). Furthermore, studies on amino acid and peptide transporters suggest that ECM fungi have a large capacity to assimilate the catabolites of extracellular proteases. Although only few transporters have fully been characterized (Nehls et al. 1999; Wipf et al. 2002; Benjdia et al. 2006), in silico analysis of the *L. bicolor* genome revealed that ECM fungi have a large gene repertoire of amino acid and oligopeptide transporters (Lucic et al. 2008).

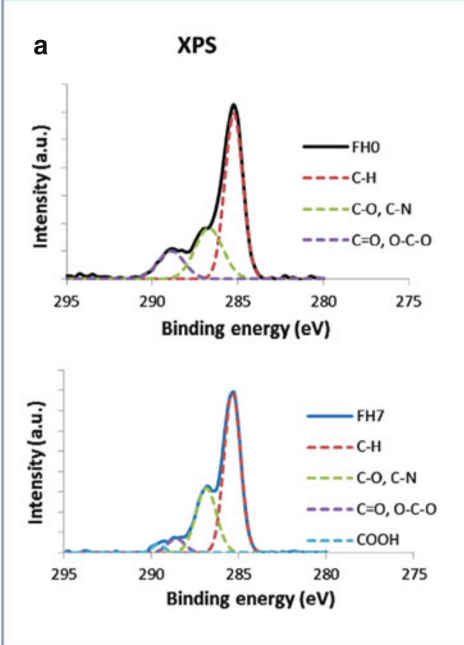
We have recently examined the proteolytic machinery expressed by *P. involutus* during the assimilation of N from various protein sources and extracts of organic matter (Shah et al. 2013). All substrates induced a similar proteolytic activity that was almost completely suppressed by the aspartic peptidase inhibitor pepstatin. However, a transcriptome analysis showed that the fungus expressed a large diversity of extracellular endo- and exopeptidases. The expression levels of these peptidases were regulated in concert with N transporters and enzymes involved in the assimilation and metabolism of the liberated peptides and amino acids.

8.6 Challenges and Future Prospects

8.6.1 Novel Spectroscopic Methods

In recent publications we have shown that infrared (IR) spectroscopy is an excellent tool to qualitatively trace the oxidative degradation of SOM induced by ECM fungi and Fenton mechanisms (Rineau et al. 2012; Rineau et al. 2013). However, from these data it is difficult to exactly pinpoint the nature of the functional groups consumed and produced during the degradation processes as well as to quantitatively estimate the extent of the oxidative reaction. These possibilities are offered by synchrotron-based techniques such as X-ray photoelectron spectroscopy (XPS) and near-edge X-ray absorption spectroscopy (NEXAFS) as indicated by new but unpublished results from our group. For instance, the high spectral resolution of synchrotron C 1s XPS data resolves classes of SOM functional groups (Fig. 8.5a), and the individual peak areas in these spectra can be used as a relative measure of the quantitative changes of SOM, which directly indicate the extent of oxidative processes. The example provided in Fig. 8.5 shows that 7 days of incubation with *P. involutus* results in a substantial increase of oxidized carbon in the form of carboxyl groups.

We also know from recent progress in soil science that molecular structure alone does not control SOM stability. Instead, the persistence of SOM arises from complex interactions between SOM and the biological and physicochemical environment (Schmidt et al. 2011). Furthermore, SOM itself is a complex mixture consisting of relatively small molecules that associate with each other in supramolecular aggregates. These aggregates are stabilized by hydrophobic interactions and hydrogen (H) bonding, and their properties are determined by all of the constituents and their associations. It follows that to understand SOM degradation by unifying the gene expression information of the fungi with information about the molecular structure and properties of the SOM, spectroscopic probes are needed to determine SOM properties at high spatial resolution and under in situ conditions. More specifically, techniques are required that will allow, at high spatial resolution (from micrometers to nanometers), to follow molecular-scale changes in SOM in terms of chemical states and relative abundances of atomic species and to precisely



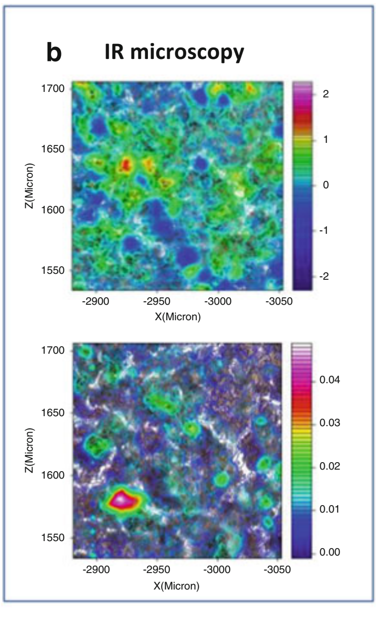


Fig. 8.5 Synchrotron- and IR-based methods for characterizing SOM (C. Nicolas, A. Tunlid and P. Persson, unpublished results). (**a**) Carbon (1 s) spectra obtained by XPS analysis of organic matter extracts of forest litter before (FH0) and after 7 days of incubation with *P. involutus* (FH7). Measured data (*full line*) were fitted by Gaussian curves (*dashed line*). (**b**) IR microscopy images of a thin layer of SOM, obtained by plotting the intensity of second derivative peaks representing carbohydrates (*top*) and carbonyl groups (*bottom*). The images indicate a physical separation between regions rich in carbohydrate and carbonyl, respectively

identify the reaction pathways of the degradation process. Such possibilities are now emerging primarily due to development of spectroscopic imaging techniques at synchrotron radiation sources but also by the use of conventional light sources; an example of IR imaging of SOM using conventional IR light is shown in Fig. 8.5b. Some studies have already demonstrated the importance and potential of analyzing the chemical structures of intact SOM at high sensitivity and spatial resolution using synchrotron methods (Lehmann et al. 2008). Further developments that allow dynamic processes to be assessed are likely to revolutionize the understanding of the molecular interactions between SOM, mineral particles, and microorganisms in the soil and how these interactions affect the stabilization of soil C. In addition to XPS, NEXAFS, and IR spectroscopy already mentioned, scanning transmission X-ray microscopy (STXM) and Raman spectroscopy will play important roles in this area. Finally, the continued development of synchrotron sources with increasing brilliance and coherence will certainly make important contributions to the understanding of SOM degradation and stabilization.

8.6.2 Development of Novel Biomarkers

There is accumulating evidences that mycorrhizal fungi have a key role in important ecosystem processes such as C cycling and nutrient mobilization. However, there is a lack of methods that can measure the functional activities of these organisms in the field. The current biomarkers include hydrolytic and oxidative enzymes (Pritsch and Garbaye 2011), yet the understanding of the involvement of such enzymes in decomposing and nutrient cycling processes is limited. For example, several of these enzymes are not expressed during the decomposition of litter material by ECM fungi (Rineau et al. 2012). Moreover many of the commercial substrates used are not relevant for inferring the decomposition of the complex C sources and nutrients that are present in SOM. The decomposing activity of soil fungi is also analyzed on the basis of transcriptional gene expression (Kellner et al. 2010). A major challenge of using such markers is that many of them belong to large gene families, and only a few of their members may have a role in the decomposition of SOM (Rineau et al. 2012).

The combination of spectroscopic methods and transcriptomic analyses offers new possibilities to identify specific genes or chemical signatures that can be used as biomarkers for probing the decomposing activity of microorganisms in soils. Statistical methods can be used to correlate gene expression levels with modifications in the substrate. Recently, such an approach was used to show that in *P. involutus* the expression levels of specific sets of genes encoding lignocellulose and protein-degrading enzymes can be correlated with specific modifications of the plant-litter material (Rineau et al. 2013). A similar experimental system can be used for analyzing the decomposing activities in a wide range of mycorrhizal and saprophytic litter fungi. Through systems biology approaches, the changes in spectra—and thus in the chemical modifications of the SOM—can be correlated with changes in gene expression. In the long run, it should be possible to generate models that will predict how a given change in transcriptional levels of a smaller set of biomarkers would change the chemical composition of the substrate. A challenge in analyzing such biomarkers in the field is to identify the relative contribution of saprotrophic mycelium *vs* the symbiotic one. An approach to tackle this issue is the use of ingrowth mesh bags that are either accessible or inaccessible to ECM fungi, using cylinders to block the connection between tree roots and fungi colonizing the mesh bags (Wallander et al. 2011). The contribution of ECM mycelium can then be deduced by subtracting the activity within the cylinder to the activity in other mesh bags. The mesh bags can be amended with plant-litter material or specific litter material. The decomposition of this material can be followed by measuring either the expression levels of specific transcripts or chemical modifications of the substrate that are proxys for free radical production.

The use of high-throughput sequencing and the next-generation molecular tools will provide ecologists with unprecedentedly large datasets describing the diversity and composition of microbial and fungal communities. Associating genes to function is of major importance for translating the growing sequence data into

ecologically meaningful traits including nutrient cycling and decomposition of organic material. Studies in model systems with reduced complexity that is amenable to integrate genomic and functional analysis offer unparalleled opportunities to dissect such associations. The increasing availability of sequenced fungal genomes will make it possible to identify which taxa have the genetic potential to produce the key enzymes or metabolic machinery that contribute to the ecological process. Then the genetic potential for enzyme production could be linked to phylogeny. Such information will be important for predicting how microbial community composition will relate to ecosystem functioning (Martiny et al. 2013; Zimmerman et al. 2013).

Acknowledgements The work has been supported by grants from the Swedish Research Council (VR), the strategic research program Biodiversity and Ecosystem Services in a Changing Climate (BECC) and the Danish Agency for Science and Technology, and the Research Foundation—Flanders (FWO). The genome and transcriptome sequencing of *Paxillus involutus* was funded by grants from the U.S. Department of Energy.

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Chapter 9
Cochliobolus heterostrophus: A Dothideomycete Pathogen of Maize

Benjamin A. Horwitz, Bradford J. Condon, and B. Gillian Turgeon

9.1 Biology and Plant Pathology

9.1.1 Cochliobolus heterostrophus *and Southern Corn Leaf Blight*

Cochliobolus heterostrophus, a pathogen of maize, belongs to the Dothideomycetes, a large and ecologically diverse class within the Ascomycota. The biology of *C. heterostrophus* and its history as a plant pathogen and genetic model system have been reviewed recently (Turgeon and Baker 2007); a brief summary is given here. The Dothideomycete class currently consists of 12 orders. *C. heterostrophus* is a member of the Pleosporales, which includes aggressive pathogens of both monocots and dicots, as well as saprobes. Among pathogenic *Cochliobolus* spp. are several species that are exquisitely specific to cereal hosts, and their tight phylogenetic relationship suggests that diversification happened quite recently, i.e., <17MYA (Ohm et al. 2012) (Fig. 9.1). Seven *Cochliobolus* sequences, representing six species, are available through the Joint Genome Institute's (JGI) portal (Grigoriev et al. 2012), and several were included in a large comparative study of Dothideomycete genomes (Fig. 9.1) (Ohm et al. 2012).

C. heterostrophus, found as two races (O and T), causes Southern Corn Leaf Blight (SCLB) (Yoder 1988; Turgeon and Yoder 2000; Turgeon and Baker 2007). *C. heterostrophus* race T produces the host-specific polyketide, T-toxin, which in combination with URF13, a mitochondrial protein found in Texas male-sterile

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*,
Soil Biology 36, DOI 10.1007/978-3-642-39339-6_9,
© Springer-Verlag Berlin Heidelberg 2013

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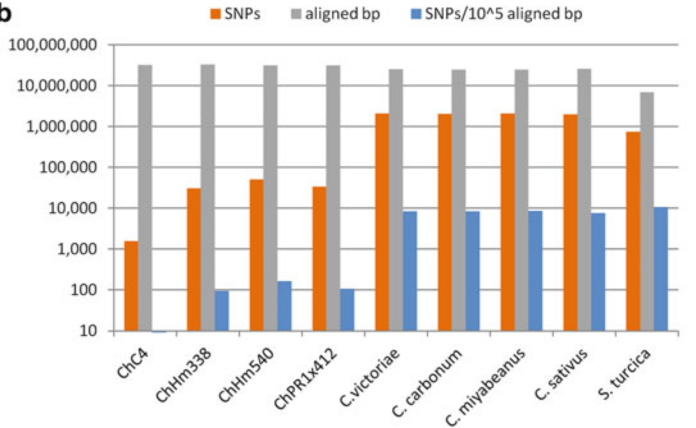
Cochliobolus heterostrophus
Cochliobolus heterostrophus
Cochliobolus sativus
Setosphaeria turcica
Alternaria alternata
Alternaria brassicicola
Pyrenophora tritici-repentis
Pyrenophora tritici-repentis
Pyrenophora teres
Leptosphaeria maculans
Leptosphaeria maculans
Phaeosphaeria nodorum/Stagonospora nodorum
Phoma herbarum
Ascochyta pisi
Didymella exigua
Corynespora cassiicola
Ascocratera manglicola
Aigialus grandis
Mytilinidion scolecosporum
Mytilinidion mytilinellum
Cenococcum geophilum
Cenococcum geophilum
Rhytidhysteron rufulum
Rhytidhysteron rufulum
Hysterium angustatum
Hysterium pulicare
Aliquandostipite khaoyaiensis
Botryosphaeria dothidea
Macrophomina phaseolina
Guignardia bidwellii
Coniosporium apollinis
Coniosporium apollinis
Hysteropatella clavispora
Patellaria atrata
Mycosphaerella populorum
Mycosphaerella populicola
Cercospora zeae-maydis
Mycosphaerella fijiensis
Passalora fulva/Cladosporium fulvum
Mycosphaerella pini/Dothistroma septosporum
Zymoseptoria tritici/Mycosphaerella graminicola
Zymoseptoria tritici
Mycosphaerella punctiformis
Baudoinia compniacensis
Teratosphaeria jonkershoekensis
Hortaea werneckii
Cladosporium herbarum
Cladosporium cladosporioides
Scorias spongiosa
Capnodium coffeae
Dothidea sambuci
Aureobasidium pullulans
Elsinoë centrolobi
Elsinoë phaseoli
Myriangium duriaei
Trypethelium nitidiusculum
Astrothelium cinnamomeum
Venturia inaequalis
Apiosporina collinsii
Tyrannosorus pinicola
Phaeotrichum benjaminii
Trichodelitschia bisporula
Microthyrium microscopicum
Tubeufia paludosa
Tubeufia cerea
Opegrapha dolomitica
Simonyella variegata

Pleosporaceae
Pleosporineae
Pleosporales
Mytilinidiales
Pleosporomycetidae
Hysteriales
Jahnulales
Botryosphaeriales
Patellariales
Mycosphaerellaceae
Dothideomycetes
Capnodiales
Dothideomycetidae
Dothideales
Myriangiales
Myriangiales
Trypetheliales
Venturiales
Arthoniales

425 400 375 350 325 300 275 250 225 200 175 150 125 100 75 50 25 0
Dothideomycetes & Arthoniomycetes
Capnodiales
Pleosporales
Mycosphaerellaceae
Pleosporineae
Pleosporaceae
Time (MYA)



maize (Dewey et al. 1988), results in toxin-associated chlorosis around necrotic lesions, followed by severe wilting and often death of the entire leaf. T-cytoplasm maize was used because its male sterility facilitates easy cross hybridization; its mitochondria, however, are sensitive to T-toxin (Lim and Hooker 1971; Dewey et al. 1988; Turgeon and Baker 2007). SCLB is not usually a very destructive disease, causing lesions on the leaves like those shown in a laboratory experiment (Fig. 9.2). In 1970, however, a severe epidemic began in Florida and spread throughout the eastern USA and as far west as Nebraska and eastern Texas and north to Canada. The severe disease coincided with the emergence of toxin-producing race T of the SCLB pathogen. Farmers no longer grow T-cytoplasm maize, and other male-sterile genetic backgrounds are now used to produce maize hybrids (Ullstrup 1972; Levings 1993; Weider et al. 2009). Three race T and two race O strains, including highly inbred as well as field isolates, have been sequenced (Condon et al. 2013). *C. heterostrophus* represents the Dothideomycetes in this volume for several reasons. It was one of the very first filamentous ascomycete plant pathogens sequenced, in the early 2000s by Celera Genomics for the Torrey Mesa Research Institute (TMRI)/Syngenta (Kroken et al. 2003). Moreover, it is a good genetic model. Not being a devastating plant pathogen, *C. heterostrophus* has an advantage for basic research: standardizing molecular genetic work on brown spot of rice, for example, would entail obvious difficulties in sharing material. A high frequency of homologous recombination and the development of tools for molecular and classical genetics (Turgeon et al. 1985, 2008; Catlett et al. 2003; Debuchy and Turgeon 2006) make this an attractive system. The following discussion in this section follows the life cycle and the disease cycle, beginning with sporulation.

9.1.2 Asexual and Sexual Sporulation

Asexually produced *C. heterostrophus* spores (conidia) are the main type of propagule infecting plants and are spread by wind and rain. *C. heterostrophus* conidiates on the plant, allowing re-dispersal. In the laboratory, colonies produce few or no spores when grown in total darkness. In continuous light conidiation is profuse, and under light/dark cycles rings of conidia are produced, and melanin production is also periodic (Wu et al. 2012) (Fig. 9.2). It is not yet clear whether an endogenous circadian rhythm underlies this periodic development. In the field, under conditions favorable to the pathogen, the disease cycle from germination to conidiation can be completed in a week.

Fig. 9.1 Sequenced genomes of *Cochliobolus* and other Dothideomycetes. (**a**) Phylogeny and estimated divergence of the Dothideomycetes. From Ohm et al. (2012); sequenced species (end 2012) are indicated by an *asterisk*. (**b**) SNPs relative to the reference genome C5, in *C. heterostrophus,* other *Cochliobolus* species, and *S. turcica*. Data are plotted from Table 3A of Condon et al. (2013)

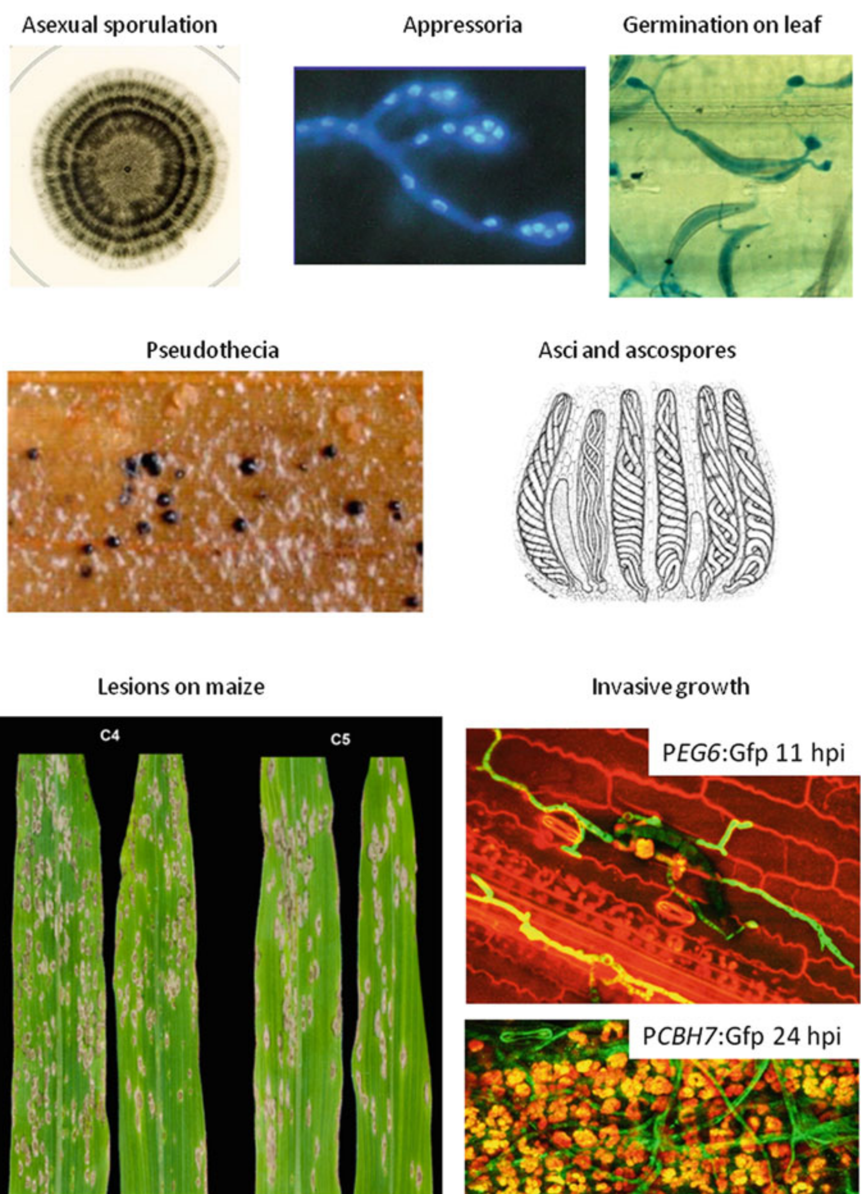


Fig. 9.2 Biology and pathogenicity of *C. heterostrophus*. *Left to right* and *top to bottom*: Asexual sporulation and melanin production result in *dark rings* on a colony growing under 8 h *light*, 16 h *dark cycles* (photo: Kent Loeffler and B.A.H.). This species forms small appressoria when conidia are germinated on a glass slide; nuclei in hyphae and the appressoria formed at their tips are visualized here by DAPI staining and fluorescence microscopy (photo: Sophie Lev). Conidia germinating on a maize leaf were stained with *lactophenol blue*; note bipolar germination and formation of appressoria on the leaf surface (photo: Sophie Lev). Pseudothecia (the filamentous hyphal network enclosing the developing asci is formed from vegetative, haploid mycelium of the "female" parent) developed on a senescent maize leaf on which an albino strain (*MAT1-1*) met a wild-type strain (*MAT1-2*); note *white* (albino) and *black* (WT melanized) pseudothecia. Asci and

C. heterostrophus is heterothallic, with mating type defined by the *MAT1* locus which exists in one of two alternate forms (idiomorphs) known as *MAT1-1* and *MAT1-2* (Turgeon et al. 1993). Mating is apparently rare in the field, but profuse formation of pseudothecia can be observed easily in the lab. To obtain the sexual stage, two strains of opposite mating type are allowed to meet on the surface of an autoclaved senescent maize leaf (Fig. 9.2). The developmental program leading to pseudothecia, meiosis, and formation of ascospores depends on the *MAT1* transcription factors (Debuchy and Turgeon 2006; Martin et al. 2010). The signaling hierarchy acting upstream of the transcription factors remains to be elucidated. It is clear that heterotrimeric G proteins, MAPK (Fig. 9.3) and two-component sensor kinases, and response regulators participate at several stages. Many signaling mutants are female-sterile but are competent as males and can still be crossed to a female-fertile partner of opposite mating type (Horwitz et al. 1999; Lev et al. 1999; Igbaria et al. 2008; Oide et al. 2010).

9.1.3 Germination of Conidia and Initial Development

Moisture activates a very rapid adhesion mechanism. Extracellular matrix secreted from the tips of the conidia allows them to stick tightly to the surface of maize leaves. This adhesion can be demonstrated in vitro: by 20 min after placing a spore suspension on a glass slide, they can no longer be easily washed off (Braun and Howard 1994). The large, multicellular conidia germinate on the leaf within about 4 h, usually from both ends. The strict polarity that is a hallmark of filamentous growth is lost, and some of the germinating hyphal tips differentiate into small appressoria (Fig. 9.2, top right). The bipolar germination pattern is reflected in one of the earlier names of the anamorph, *Bipolaris maydis*. Mutants in the MAPK gene, *CHK1*, fail to produce conidia (Lev et al. 1999), while loss of the cell-integrity MAPK *MPS1* prevents conidiation, yet the hyphal tips form appressoria on a glass or plastic surface (Igbaria et al. 2008). Nevertheless, the two signaling pathways defined by these kinases share some co-regulated target genes (Igbaria et al. 2008), suggesting further, genome-wide, experiments to find more such targets of combined regulation by the Chk1 and Mps1 MAPK pathways. Mutants in one of

Fig. 9.2 (continued) ascospores, as drawn by Drechsler (1925): note the elongated, filamentous ascospores within the asci. Symptoms, on *N*-cytoplasm maize line W64AN, caused by spraying a conidial suspension of each of the two isogenic, sequenced laboratory strains (Fig. 9.1) C4 and C5 onto 3-week-old maize plants, followed by overnight incubation in a mist chamber (photos: 5 dpi, Dongliang Wu and Kent Loeffler). Invasive growth visualized by confocal fluorescence microscopy of transgenic strains, in which Gfp expression was driven by the promoters of two cellulase genes, *EG6* and *CBH7* (Lev and Horwitz 2003); in the *upper panel* (11 h hpi), strong background fluorescence (*red channel*) is mainly from the underlying chloroplast-containing mesophyll layers, while in the *lower panel* (24 hpi), *green fluorescent* mycelia are seen between mesophyll cells, in which chlorophyll fluorescence has faded as necrosis progressed (images: Sophie Lev)

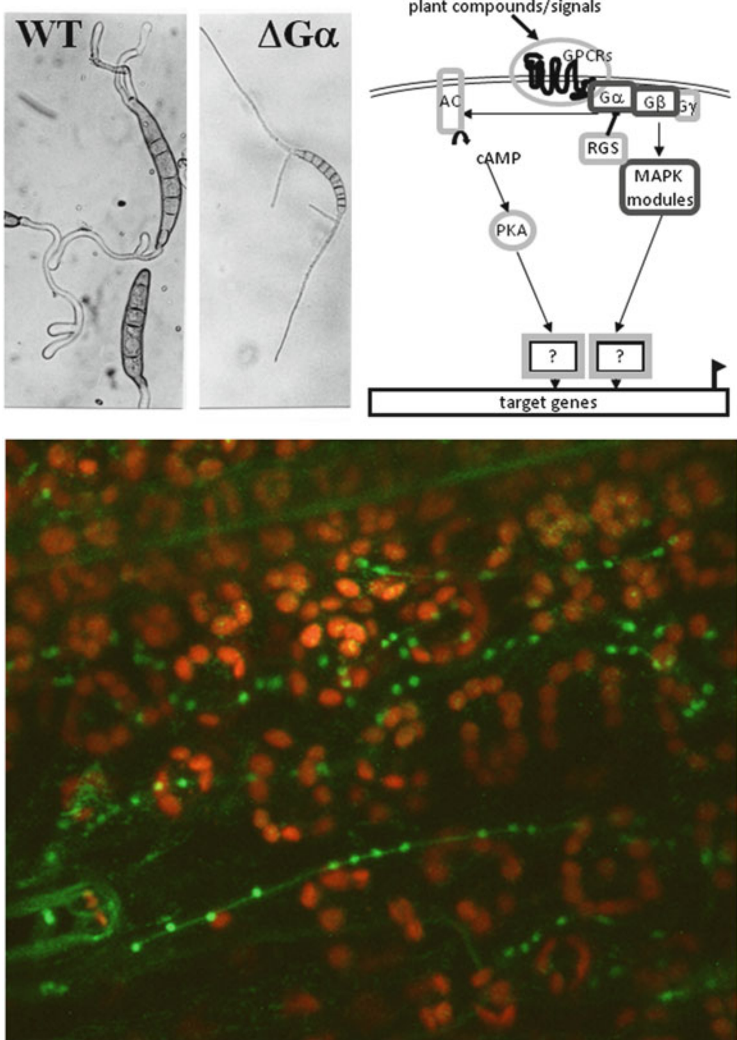


Fig. 9.3 Signal transduction. Top, scheme of a part of the signaling network. Existing mutants are shown in *darker frames*. *Left*, development on a glass slide of WT (strain C4) or a loss of function mutant in the G protein Gα subunit gene *CGA1* (Horwitz et al. 1999); note formation of appressoria by the WT and straight polar growth of the mutant (photos: B.A.H.). *Bottom*, visualization of the activation of one of the transcription factors involved in processing signals from the environment in *C. heterostrophus*. The redox-sensitive transcription factor ChAp1 is retained in the nuclei of hyphae growing within a maize leaf, as detected by Gfp::ChAp1 fluorescence, in response to oxidant or other plant signals (Lev et al. 2005). *Red autofluorescent ellipsoids* are mesophyll chloroplasts; part of a pair of stomatal guard cells is also visible at the *lower left* (*green autofluorescence*). Imaged at 12 hpi (M. Ronen, B.A.H. and Maayan Duvshani-Eshet)

the three G protein alpha subunit genes, *CGA1*, conidiate normally but upon germination show a clear phenotype: instead of forming appressoria, hyphae continue to grow in a straight path, apparently oblivious to the presence of a hard surface (Fig. 9.3). Surprisingly, *cga1* mutants are able to infect maize (Horwitz et al. 1999), though the mutant phenotype depends on the host leaf age (Degani et al. 2004).

9.1.4 Entry into the Leaf

Appressoria are not necessary for penetration into the leaf (Horwitz et al. 1999). Entry occurs with the help of enzymes that break down the cuticle and cell walls, or via the stomatal pores. *C. heterostrophus* thus enters the leaf using a strategy that is different from some other foliar pathogens like rice blast (Chap. 4). Although appressorial turgor pressure generation does not seem to be important, *C. heterostrophus* appressoria detect the plant surface. An endoglucanase promoter drives strong expression of a Gfp reporter gene already at the appressorial stage and during invasive growth (Lev and Horwitz 2003) (Fig. 9.2). This suggests that the appressorium could have an exploratory, signaling role rather than directly "breaking in" through the epidermis. Loss of polar tip growth occurs on plastic or glass. The plant likely supplies a variety of additional signals, but it is not clear at what stage they act or through what mechanism. The redox-sensing transcription factor ChAp1 (ortholog of yeast YAP1) becomes localized to nuclei upon exposure to oxidative stress in *C. heterostrophus* and other plant pathogens (Lev et al. 2005; Molina and Kahmann 2007; Kim et al. 2009; Temme and Tudzynski 2009; Huang et al. 2011). *C. heterostrophus* appressoria formed in water on a glass surface do not activate ChAp1, but Gfp::ChAp1 localizes to the nucleus when conidia germinate on the leaf surface. Plant extracts, even if treated with catalase to remove H_2O_2, are able to activate *ChAP1* (Lev et al. 2005). Phenolic compounds, ubiquitous in plants and encountered early on by the invading pathogen, promote nuclearization of ChAp1, but cannot induce its antioxidant target genes (Shanmugam et al. 2010; Shalaby et al. 2012). Nevertheless, plant extract has both activities, suggesting that more active plant molecules need to be isolated.

9.1.5 Invasion and Virulence

Once inside the leaf, the fungus grows between the mesophyll cells which at first appear to remain viable (Figs. 9.2 and 9.3), but cell death can be observed as early as 6 hpi. *C. heterostrophus* is thus considered a necrotroph with no (hemi) biotrophic stage, though a necrotrophic lifestyle cannot always be sharply delineated from a hemibiotrophic one (Oliver and Solomon 2010). The hyphae initially tend to run parallel to the vascular bundles but later branch and invade

tissue in all directions (Lev and Horwitz 2003) (Fig. 9.2). By 12 h, the fungus produces visible lesions. Some of the same signaling pathways needed for development (Fig. 9.3) also participate in attack on the plant: these include MAPK (Lev et al. 1999; Igbaria et al. 2008), G protein (Ganem et al. 2004), and two-component signaling pathways (Oide et al. 2010). The developmental regulators ChLae1 and ChVel1 (Wu et al. 2012), like their *Aspergillus* orthologs (Chap. 5), modulate secondary metabolite biosynthesis and development in response to darkness or light. Loss of these regulators also affects sexual and asexual sporulation (Wu et al. 2012).

The infection court presents an unfriendly environment to the invading pathogen. Plants fight infection with an oxidative burst. Nevertheless, necrotrophs actually thrive in an oxidant environment, as shown first for *Botrytis cinerea* (Govrin and Levine 2000; Heller and Tudzynski 2011). This may explain the apparent paradox that ChAp1 is retained in the nucleus during invasive growth (Fig. 9.3) yet is nonessential for virulence, at least on the maize cultivar studied by Lev et al. (2005). The *Botrytis cinerea* YAP1 ortholog is essential for oxidative stress response in vitro, yet does not induce its antioxidant targets during infection (Temme and Tudzynski 2009). This suggests some very special regulatory circuits used by necrotrophs in their adaptation to the host environment. Induction of ChAp1 targets has not yet been followed *in planta* in *C. heterostrophus*. Stress response machinery other than ChAp1 is, however, required for virulence. Loss of the *C. heterostrophus* gene encoding the stress-activated MAPK (Hog1) or its upstream two-component signal receiver (response regulator Ssk1) decreases virulence (Oide et al. 2010) (Fig. 9.3). The transcriptional regulators ChLae1 and ChVel1 are needed to tolerate oxidative stress and are required for full virulence (Wu et al. 2012).

9.2 Genome

9.2.1 Statistics and SNPs

The Joint Genome Institute (JGI, CA) has sequenced two inbred laboratory strains and three field isolates, as well as one strain each of five more species of the genus, *C. victoriae, C. carbonum, C. miyabeanus, C. lunatus,* two strains of *C. sativus*, and one member of the closely related genus Setosphaeria, *S. turcica* (http://genome.jgi.doe.gov/programs/fungi/index.jsf). For a complete discussion, see Condon et al. (2013). The laboratory race O strain C5, inbred by six backcrosses (Leach et al. 1982), is the reference strain to which the other members of the genus are compared. With only 68 scaffolds, the C5 assembly is most resolved. The *Cochliobolus* genome sizes of 32–38 Mb are typical for Ascomycetes and do not suggest any duplications of large regions. The numbers of gene models, 11,700–13,200, are also typical of these fungi. The *S. turcica* genome, 43 Mb, is a little larger than the six

representatives of *Cochliobolus*, but the predicted transcriptome is encoded by 11,700 genes. Single-nucleotide polymorphisms (SNPs) provide a good indication of the extent to which closely related genomes differ. Software (Kurtz et al. 2004) is available to call (identify) SNPs. The two inbred laboratory strains, C4 and C5, are an obvious case in point: they differ at the *Tox1* locus (loci, actually, see discussion below) and at the *MAT1* locus where C5 has the *MAT1-1* idiomorph and C4 the *MAT1-2* idiomorph. Other than these two major differences, C4 and C5 are nearly isogenic, confirming the assumption that guided work on these strains over the years: only 1,584 SNPs differentiate C4 from the reference C5 (Fig. 9.1). Looking outside *Cochliobolus*, only 16 % of the *S. turcica* genome could be aligned to the *C. heterostrophus* reference strain C5. The aligned regions, nevertheless, contain fewer SNPs than some of the other species within *Cochliobolus* (Fig. 9.1). All three *C. heterostrophus* field isolates differed from C5 by an order of magnitude more SNPs than the inbred C4 (Fig. 9.1). The race O field strain has more SNPs than the two race T strains, in agreement with RFLP data implying that race T arose from race O (Yang 1995; Yang et al. 1996; Kodama et al. 1999). Looking at other members of *Cochliobolus*, the alignment of reads to C5 remains high considering that they are distinct species, but the numbers of SNPs are in the millions (Fig. 9.1).

The phylogeny and evolutionary view (Fig. 9.1) provide the starting point for detailed investigation of classes of genes that could provide some understanding of why one species is a pathogen and another a saprobe, has a narrow or wider host range, or particular developmental patterns. This is no easier for *Cochliobolus* than for other fungi, but extensive previous work suggested, as a first choice, for necrotrophs secondary metabolite biosynthesis genes and small secreted cysteine-rich proteins (SSCPs) for hemibiotrophs (Condon et al. 2013). We know from previous work on *C. heterostrophus* that secondary metabolites are important for virulence and stress resistance [e.g., T-toxin, a family of polyketides biosynthesized by polyketide synthases (Turgeon and Baker 2007), and siderophores, biosynthesized by nonribosomal peptide synthetases (Oide et al. 2006, 2007)]. We also know from classic (Doubly et al. 1960; Joosten and de Wit 1999) and recent studies on "avirulence" genes, now referred to as pathogen effectors [see Win et al. (2012)], that SSCPs are key words (or weapons) in the pathogen-host molecular dialog. The possibilities for bioinformatic study beyond these two groups are very large. Having focused, though, on these two groups, we proceed to discuss them briefly, along the lines described in the *Cochliobolus* genome paper (Condon et al. 2013).

9.2.2 Secondary Metabolites

9.2.2.1 Nonribosomal Peptide Synthases

These multimodular enzymes produce an amazing variety of peptide secondary metabolites. Some have conserved functions. Acquisition of iron, for example, is a

common need for all microorganisms. Mutants deleted for all genes encoding Nonribosomal Peptide Synthases (NRPS) (*NPS*) in *C. heterostrophus* were constructed by introducing a selectable marker in place of each gene, by homologous integration (Lee et al. 2005; Bushley and Turgeon 2010). Only one, *NPS6*, resulted in a virulence phenotype when compared to wild type (Lee et al. 2005; Oide et al. 2006). Identification of the product as the extracellular siderophore coprogen linked siderophore-mediated iron uptake to virulence in *C. heterostrophus* and other plant pathogens, including the wheat pathogen *Fusarium graminearum* and the Arabidopsis pathogen *Alternaria brassicicola* (Oide et al. 2006). The evolutionary relationships of NRPS in *Cochliobolus* species and *S. turcica* were studied using the fungal AMP-binding (AMP) domain hidden Markov model (HMM) developed by Bushley and Turgeon (2010). Phylogenetic trees were built based on a comparative NRPS AMP domain inventory. Within Cochliobolus, conservation of NRPSs is almost complete, while at the genus level, only seven of the 14 NRPS in *C. heterostrophus* reference strain C5 were conserved. When the related maize pathogen *S. turcica* was added to the comparison, this number dropped to six of the 14. The conservation within species, and its loss when looking outward at the species and genus levels, suggests that NRPSs and consequently the peptide metabolites have diversified rapidly, along with the choice of host and niche (Condon et al. 2013).

9.2.2.2 Polyketide Synthases

Polyketide synthases (PKSs) are responsible for the biosynthesis of secondary metabolites, including some host-specific toxins. Like NRPS, PKSs are large, multidomain enzymes. 23 and 25 *PKS* genes are annotated in the C5 and C4 genomes, respectively (Kroken et al. 2003; Condon et al. 2013). Using the PFAM ketosynthase domain (KS) HMM as a query to search for orthologs, it was found that most of the PKSs annotated in the reference genome are conserved across all *C. heterostrophus* strains (Condon et al. 2013). There are some exceptions: field strain Hm338 lacks PKS16, PKS25 is missing from strain Hm540, and PKS13, a pseudogene, is found only in strains C5 and C4. *PKS1* and *PKS2,* genes required for biosynthesis of T-toxin, are present in, and only in, T-toxin producing *C. heterostrophus* race T strains.

As mentioned above, the polyketide T-toxin is responsible for the supervirulence of race T against T-cytoplasm maize. Production of T-toxin requires two unlinked loci, *Tox1A* and *Tox1B*, which have been mapped to the breakpoints of a reciprocal translocation of two race T chromosomes (12;6, 6;12) relative to their counterparts in race O. *Tox1A* and *Tox1B* belong to 1.2 Mb of DNA that is absent from T-toxin nonproducers. Loss of either *PKS1* or *PKS2* eliminates the production of T-toxin (Baker et al. 2006; Inderbitzin et al. 2010). Known *Tox1* genes, such as *PKS1*, are located on very small scaffolds (~25 kb) in race T strains C4, Hm338, and PR1x412. These small scaffolds could not be further assembled due to the repetitive and AT-rich nature of the *Tox1* locus (Condon et al. 2013).

As with the NRPSs discussed in the preceding section, conservation of *PKS* genes is not as high across *Cochliobolus* species as between *C. heterostrophus* strains and still less when *S. turcica* is added to the phylogeny. Seven PKSs out of the 23 in reference strain C5 are conserved in all *Cochliobolus* species and *S. turcica*. Of these, the only one responsible for a known metabolite is PKS18, which starts the biosynthesis of DHN melanin. Thus, there are conserved PKSs that are likely to synthesize unknown, but important, products.

9.2.3 Small Secreted Cysteine-Rich Proteins

The gene model catalogs for each species were filtered for small size (<200 amino acids), at least 2 % cysteine residues, secretion signal, and lack of transmembrane domains (Condon et al. 2013). The number of SSCPs identified in this way was 141–289. The numbers would change, of course, depending on the bioinformatic cutoff criteria used, but it is clear that each strain and species has unique SSCPs. Only 1–21 were unique to a particular *C. heterostrophus* strain, providing interesting candidates for functional analyses. The highest number of unique SSCPs, 191 and 167, respectively, was found in *C. sativus* and *S. turcica*; it might not be coincidental that both these species are hemibiotrophs, perhaps needing more effectors to set up the complex initial interaction with living host cells (Condon et al. 2013). Of the 180 SSCPs in the reference *C. heterostrophus* C5 genome, 72 have no EST support. The genome thus seems to be a reservoir of "virtual" effector genes that are not expressed under conditions tested so far; *in planta* expression experiments need to be done. It is important to keep in mind that any such analysis rests on the quality of the gene models. There is no evidence for clustering of SSCP genes in the genomes as in *Leptosphaeria maculans* (Rouxel et al. 2011) or *Ustilago maydis* (Schirawski et al. 2010). They are, however, often located in proximity to or within regions identified as *C. heterostrophus* species unique (Condon et al. 2013).

9.3 Dothideomycete Soil Biology

In the field, *C. heterostrophus* overwinters in crop debris and soil, but is not really a member of the rhizosphere or soil community. Some other Dothideomycetes have closer ties to the soil. The closely related *C. sativus* causes leaf spot blotch of wheat and other cereals, and also crown (location where the stems of grasses emerge from the soil) and root rot (Kumar et al. 2002). *C. victoriae* is a crown pathogen of oats and wheat. *Setosphaeria pedicellata* is noted (http://www.cabi.org) as a root pathogen (though apparently not a serious one). *Cenococcum*, an ectomycorrhizal fungus with a wide distribution, is now firmly classified in the Dothideomycetes based on multigene phylogenetic evidence (Spatafora et al. 2012). The genus *Cenococcum* is

Fig. 9.4 Infection of maize roots by *C. heterostrophus*. *Top* (N), *C. heterostrophus* WT strain C4 is not able to infect the root of corn cv. W64-A with *N*-cytoplasm successfully. Plant roots challenged by strain C4 (WT) are shown. Image was taken 10 days after inoculation. Mock-inoculated control is shown on *right*. Inoculation of corn roots with *C. heterostrophus* was carried out as described by Dufresne and Osbourn (2001) on inoculation of rice roots with *Magnaporthe oryzae*. No clear difference was observed between the plants challenged by C4 and mock-inoculated plants, indicating that *C. heterostrophus* strain C4 is not able to infect the roots of maize cv. W64A with N-cytoplasm. *Bottom*, *C. heterostrophus* strain C4 infects the roots of maize cv. W64A with Tcms (T) successfully. Roots of corn cv. W64-A with Tcms challenged by *C. heterostrophus* strain C4 (WT). Images were taken 10 days after inoculation. Mock-inoculated controls are shown on *right*. Development of roots and shoots was severely attenuated in the plants infected by WT strain (K. Oide and B.G.T, unpublished; photos: K. Oide and Kent Loeffler)

closely related to the Pleosporales (Ohm et al. 2012) (Fig. 9.1). Thus, a mutualistic species keeps bad company (phylogenetically) with some destructive pathogens of plants. *Cenococcum geophilum* is currently being sequenced at the JGI. Comparison with related pathogens (Fig. 9.1) may shed light on what makes a symbiont different from a pathogen. From the physiological side, a first experiment has been done, along the lines that led to the discovery that rice blast can infect rice roots (Dufresne and Osbourn 2001; Sesma and Osbourn 2004) (Chap. 4) and that *Colletotrichum graminicola* can infect maize roots (Sukno et al. 2008). *C. heterostrophus* can indeed infect roots, intriguingly, only when the interaction is between race T of the pathogen and T-cytoplasm maize, where T-toxin is a virulence factor (Fig. 9.4).

9.4 Conclusions

C. heterostrophus has been a useful model to study how development, secondary metabolites, stress physiology, and signaling pathways are involved in virulence. Among the questions to be addressed in this and other systems are as follows: why a particular species can infect roots or shoots, the molecular basis of host range, and what makes the difference between a pathogen and a symbiont. The genus *Cochliobolus* and Dothideomycetes in general have been the focus of an unusually intense genomic sequencing effort. This raises the exciting possibility that these often destructive pathogens may be among the first to yield the answers to some of the most challenging questions in plant pathology.

Acknowledgements Research in our laboratories, including unpublished data shown in this chapter, was funded in part by USDA, BARD, and ISF. We thank Kent Loeffler (Cornell University) for superb photography and Maayan Duvshani-Eshet (Technion) for her assistance with confocal microscopy.

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Chapter 10
Penicillium chrysogenum: Genomics of an Antibiotics Producer

Marco A. van den Berg

10.1 Introduction

The species *Penicillium chrysogenum*[1] was originally described by Thom (1910). The conidiophore of the fungus resembles a paintbrush; hence, the genus name *Penicillium* is derived from penicillus, the Latin word for paintbrush. The species identification *chrysogenum* comes from the yellow pigment chrysogenin produced by the natural isolates. *Penicillium* has long been considered to be a member of the deuteromycetes, fungi with no known sexual state. Still, various studies identified several remnants of sexual activity in the past (Braumann et al. 2008; Hoff et al. 2008; Henk et al. 2011; Böhm et al. 2013).

Through history the fungus has also been known as *P. notatum* being documented as the source for the discovery of penicillin (Fleming 1929). Penicillin is active against gram-positive bacteria (i.e., Staphylococcus and Pneumococcus) by disrupting bacterial cell wall synthesis, i.e., by inhibition of the transpeptidase required for cross-linking of the peptidoglycan polymers. This leads to weakened cell walls and uptake of excess water, which causes cell burst. Moreover, peptidoglycan precursors accumulate due to the inhibition of transpeptidase, which in turn induces autolytic hydrolases that will digest existing peptidoglycan (Moreillon et al. 1990) without the production of new peptidoglycan. This enhances the bactericidal action of penicillin.

[1] Although Houbraken et al. (2011) demonstrated that both Fleming's isolate and the Wisconsin 54–1,255 strain are not *P. chrysogenum* but *P. rubens*, the name *P. chrysogenum* will be used throughout this chapter to avoid confusion as the *P. rubens* name is not yet used in databases, culture collections, and literature.

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*,
Soil Biology 36, DOI 10.1007/978-3-642-39339-6_10,
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The ability to produce penicillin, as well as a range of other metabolites (Table 10.1), has evolved over millions of years and is shared with several other related fungi[2] (Fig. 10.1). Producing this array of compounds is believed to give the fungus a selective advantage during competition for carbon and nitrogen. Natural isolates with the capability to grow submerged while producing penicillin were the founding strains of all current industrial derivatives. These industrial strains can produce enormous amounts of penicillins under harsh conditions in 100–200 m^3 fermenters. The penicillin biosynthetic genes, *pcbAB*, *pcbC*, and *penDE*, are physically linked, forming a cluster that is amplified in industrial production strains.

10.2 Origin and Evolution

With the long industrial use of *P. chrysogenum*, there has been an equally long debate on the evolutionary benefit of penicillin production as well as the natural habitat of this fungus. Genome sequencing makes it possible to understand genetic changes in the lineage leading to the current strains.

10.2.1 Natural Habitats

Often, *P. chrysogenum* is described as an "airborne fungus," a term that is used to describe that its conidia are spread through air towards various new habitats which the fungus can colonize. In fact, it is a quite common fungus that can be found almost anywhere in temperate, tropical as well as arctic locations (Ismail 2001; Bancerz et al. 2005; Henk et al. 2011). Fleming's isolate (1929) was obtained in the classical way: spores transported through the air in St. Mary's hospital, whereas the ancestral strain of the current production strains—NREL1951—was isolated from rotting fruit (Raper et al. 1944). Other isolates are from a range of habitats: salted food products (Samson et al. 2010), indoor environments such as archives (Roussel et al. 2012), compost heaps (Adeleye et al. 2004), and damp buildings (Andersen et al. 2011).

All *P. chrysogenum* isolates are capable of secreting a versatile range of enzymes and metabolites. In soil, fungi have to work under the most complex conditions with limited free nutrition, limited oxygen, and many competitors. Like most soil fungi, *Penicillium* can secrete various factors to liberate complexated minerals [like phosphorus by producing phosphatases, chelating compounds, and organic anions (Richardson et al. 2011)] and metals [like iron by producing a range

[2] In total 8 species of Penicillium are reported to produce penicillin in culture: *P. allii-sativi*, *P. chrysogenum*, *P. dipodomyis*, *P. flavigenum*, *P. nalgiovense*, *P. rubens*, *P. tardochrysogenum* and *P. vanluykii* (Houbraken et al. 2012).

Table 10.1 Secondary metabolites produced by *P. chrysogenum* Legend

Compound	Molecular structure	Compound class	Mode of action	Active against	Production level	Biosynthetic genes	References
PenicillinG		β-lactam antibiotic	Inhibits the formation of peptidoglycan cross-links	Both gram-positive and gram-negative organisms	~20 mg/g	Pc21g21370–Pc21g21390	García-Rico et al. (2008), van den Berg et al. (2008)
Roquefortine C		Diketopiperazine alkaloid	Cytochrome P450 inhibitor	Gram-positives	~3 μg/g	Pc21g15430–Pc21g15480	Frisvad et al. (2004), García-Estrada et al. (2011)
Meleagrin		Diketopiperazine alkaloid	Cytochrome P450 inhibitor	Gram-positives	~0.3 μg/g	Pc21g15420–Pc21g15480	Frisvad et al. (2004), García-Estrada et al. (2011), Ali et al. (2013)
Chrysogenin		(Yellow) pigment	n.a.	n.a.	~600 μg/g	n.k.	Frisvad et al. (2004), García-Rico et al. (2008)
Xanthocillins		Isocyanide derived from shikimic acid pathway	Evaluated as thrombopoietic drug	Gram-positive and Gram-negative bacteria	~100 μg/g	n.k.	Sakai et al. (2005), de la Campa et al. (2007)
Secalonic acids		Ergochrome (polyketide)	Phosphorylation in signal transduction pathway	Antifungal	n.k.	n.k.	Frisvad et al. (2004), Houbraken et al. (2011)
Questiomycin		Polycyclic iminoquinone	DNA intercalation	Gram-positive and -negative bacteria and yeasts	n.k.	n.k.	Frisvad et al. (2004)

(continued)

Table 10.1 (continued)

Compound	Molecular structure	Compound class	Mode of action	Active against	Production level	Biosynthetic genes	References
Sorbicillin/ sorbicillactone A		Alkaloid	Radical Scavenging and inhibition of lipopolysaccharide induced Production (TNF α) in human monocytes	Antibacterial; antifungal; cytotoxic	~4 mg/l	Pc21g05050–Pc21g05110	Cram (1948), Bringmann et al. (2003, 2007), Avramović (2011)
PR-toxin		Terpene (derived from Aristolochene)	Inhibits RNA and protein synthesis; DNA polymerase	Antifungal; lethal to rats, mice and cats	n.k.	Possibly Pc12g06310–Pc12g06370	Dai et al. (1993), Harris et al. (2009a)
Andrastin A, B		Meroterpenoid (mixed polyketide-terpenoids).	Protein farnesyl transferase inhibitors	Inhibiting efflux of anticancer drugs from multidrug-resistant cancer cells	n.k.	n.k.[a]	Houbraken et al. (2011)
Citrinin		Quinone methide	DNA damage via hydroxyl radicals	Bacteriocidal, antifungal and antiprotozoal mycotoxic nephropathy in livestock	530 mg/l	n.k.[b]	Devi et al. (2009)
Fungisporin		Peptide cyclo (L-phenylalanyl-D-valyl-L-valyl-D-phenylalanyl)	Inhibits metabolism	Antibacterial	n.k.	Pc13g14330 or Pc16g04690	Frisvad et al. (2004), van den Berg et al. (2008)

Production is giving either per gram of mycelial dry weight or per liter culture broth

n.a. not applicable, *n.k.* not known

[a] Andrastin building block 3,5-dimethylorsellinic acid (Geris and Simpson 2009) is produced by *Aspergillus nidulans* AusA (Ahuja et al. 2012), and the closest homologue is Pc21g05070 (32 % identity), involved in the biosynthesis of sorbicillin and derived products

[b] Closest homologue to *Monascus purpureus* PKS (Shimizu et al. 2005) is Pc21g05070 (43 % identity)

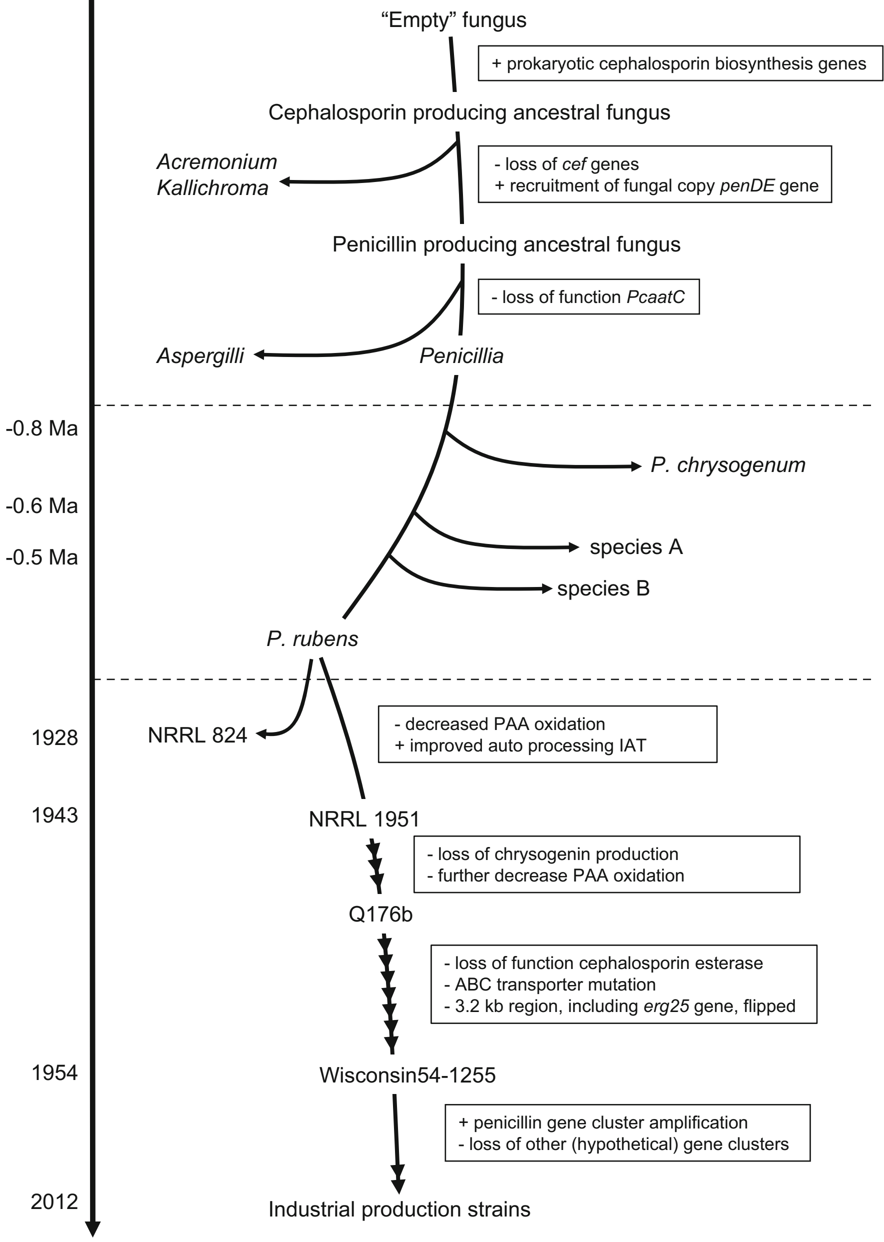

Fig. 10.1 Schematic overview of the evolutionary path of penicillin production by *Penicillium*. NRRL 824 (Fleming 1929), Fleming's isolate; NRRL 1951, first isolate for submerged growth (Raper et al. 1944); Q176b, first isolate with decreased chrysogenin (Anderson et al. 1953); Wisconsin54-1255, international laboratory strain standard (Elander 2002)

of siderophores as trihydroxymates, coprogen, and ferricrocin (Hördt et al. 2000)]. Esterases hydrolyze the iron-containing siderophores into hydroxamates to make the metal available for absorption. Also under laboratory culture conditions, this iron scavenging machinery is used in order to acquire sufficient iron for growth and penicillin production (Charlang et al. 1981; Leiter et al. 2001), as one of the key biosynthetic enzymes—isopenicillin N synthase—is dependent on iron (Burzlaff et al. 1999). Some of the siderophore synthetase genes—Pc13g05250 for ferrichrome and Pc16g03850 for triacetyl fusarinine—were readily identified from the genome (van den Berg et al. 2008). Transcription analysis using Affymetrix GeneChips suggests that the former is more important than the latter under laboratory conditions (data not shown), which corroborates earlier findings (Charlang et al. 1981).

Indirectly, fungi associated with plant roots can trigger responses in the plants' defense system, amongst others leading to a so-called state of induced systemic resistance, wherein the plant expresses several defense proteins leading to improved seed germination and growth (Chen et al. 2006; Murali et al. 2012). In fact, aqueous extracts prepared from dried commercial *P. chrysogenum* mycelium can be used as a spray or soil additive to enhance crop yield. Detailed studies showed that the extract itself did not have any antimicrobial activity and low molecular weight molecules—below 2,000 Da—are necessary to induce the defense mechanism in plants (Thuerig et al. 2006).

10.2.2 Antimicrobial Compounds

In order to eliminate competing microbes for available nutrients in the same niche, *P. chrysogenum* produces a range of secondary metabolites (Table 10.1), of which the antibacterial β-lactams are studied most extensively. However, compared to the production levels of current industrial strains, natural isolates produce only a minute amount of β-lactam antibiotics under laboratory conditions [reviewed in van den Berg (2011)]. Only a limited number of studies attempted to describe production of antimicrobial metabolites in its natural habitats as the levels are difficult to quantify. Hill (1972) clearly demonstrated penicillin production in soil and on seeds, while being counteracted by *Bacillus* species producing β-lactamases. Besides β-lactam antibiotics, *P. chrysogenum* is able to produce a range of other secondary metabolites including antifungal, antibacterial, and cytotoxic compounds (Table 10.1). For some products, the biosynthetic gene clusters have been identified. Interestingly, in the penicillin production strains, these other secondary metabolite gene clusters seem to be downregulated. Gene transcription of at least three clusters is induced under non-penicillin-producing conditions (Table 10.2).

The genome also contains remnants of other secondary metabolites, not reported to be produced by *P. chrysogenum*. The gene clusters for viridicatumtoxin and griseofulvin were most likely lost during evolution while being retained in

Table 10.2 Secondary metabolite gene clusters induced under non-penicillin-producing conditions (Harris et al. 2009a; Veiga et al. 2012a)

Contig	Gene ID's	Gene number	Putative function
Pc06	Pc06g02040–Pc06g02100	7	Unknown
Pc12	Pc12g06310–Pc12g06400	11	Involved in aristolochene biosynthesis
Pc21	Pc21g05060–Pc21g05110	6	Involved in the biosynthesis of sorbicillins and derivatives

P. aethiopicum (Chooi et al. 2010). The genes for melanin are dispersed over the genome (Pc21g16000, Pc21g16380-Pc21g16440, and Pc06g01310), while in black fungi, they are clustered and functional (Woo et al. 2010). Interestingly, the main PKS-encoding gene (*alb1* in *P. marneffei*; Woo et al. 2010) lost a copy during strain improvement: 2 gene copies can be found in Wisconsin54-1255 but only one in DS17690 (van den berg et al. 2008). Moreover, expression of the putative scytalone dehydratase (*arp1* in *P. marneffei*; Woo et al. 2010) was lost during strain improvement (Jami et al. 2009b). Both observations could explain why spores of the industrial strains are less intense green than the Wisconsin54-1255 strain.

In contrast to what has been reported, *P. chrysogenum* does not produce the carcinogenic mycotoxin sterigmatocystin (Rank et al. 2011). Furthermore, it has been suggested that some isolates produce fumigaclavine (Kozlovsky et al. 1998; Zhelifonova et al. 2010) and ochratoxin A (Gherbawy et al. 2012). However, this is not in line with detailed studies by Frisvad et al. (2004). Still, the genome might encode enzymes that could have played a role in synthesizing these compounds: the *P. chrysogenum* homologue of 6-methylsalicylic acid synthase, Pc22g08170, is 60 % identical to the PKS of P. *nordicum* involved in ochratoxin A biosynthesis (otapksPN; Karolewiez and Geisen 2005). Fumigaclavine is an ergot alkaloid, which biosynthesis starts with dimethylallyltryptophan synthase (dmaW) as reported for P. commune [reviewed in Wallwey and Li (2011)]; the *P. chrysogenum* homologue is Pc21g15430. Therefore, it could very well be that specific isolates from specific niches could produce these compounds.

In addition to metabolites, Penicillia produce a range of antimicrobial proteins, which can induce apoptosis-like cell death in sensitive fungi (Leiter et al. 2005). The most studied example is PAF (Pc24g00380), a cysteine- and lysine-rich antifungal protein (Marx et al. 1995). This small (6.25 kDa) protein is highly stable through a broad range of pH (1.5–11), heat (60 min, 80 °C), and proteases. Interestingly, PAF-producer *P. chrysogenum* is resistant through an unknown mechanism, but closely related fungi as *Aspergillus* are sensitive. Metabolically active cells take up the protein via an endocytosis-like process which triggers a very extensive signaling pathway negatively influencing several elements in sensitive cells [reviewed in Hegedus et al. (2011)]. The genome encodes more putative small antifungal proteins. One of them (Pc21g12970) is homologous to the bubble protein (BP) of *P. brevicompactum*, the mycophenolate-producing fungus, which inhibits *Saccharomyces cerevisiae* in a dose-dependent manner (Seibold et al. 2011). However, under laboratory conditions, this gene is not transcribed (van den Berg et al. 2008). A third

ORF, Pc12g08290, is closely related to the PAF protein. Interestingly, expression of both PAF and Pc12g08290 is repressed under penicillin-production conditions when the phenylacetic acid side-chain precursor is present (van den Berg et al. 2008), whereas this repression does not occur in strains lacking the whole penicillin biosynthetic cluster (Harris et al. 2009a).

Fungi also have to protect themselves against the molecules produced by other microbes. Most likely, many of the 830 putative transporters of *P. chrysogenum* (van den Berg et al. 2008) will have a crucial role in this, although reported examples are limited to fluconazole and cycloheximide resistance proteins, induced by glucose (Castillo et al. 2005).

10.2.3 Biochemical Arsenal

As many saprophytic fungi, *P. chrysogenum* is able to grow on lignocellulosic material (Mishra et al. 1979; Rodríguez et al. 1994) via a wide range of secreted enzymes. In addition, proteome studies confirm the presence of the biochemical weaponry, including proteases, phosphatases, and oxidases, required for plant biomass degradation (Jami et al. 2009a). Besides enabling growth on plant material, many of these enzymes are useful in industrial applications as food processing and biofuel production. The *P. chrysogenum* genome harbors 174 ORF's encoding putative enzymes involved in plant polysaccharide degradation (van den Brink and de Vries 2011), allowing the fungus to grow on hemicellulosic, cellulosic, as well as on lignin-containing materials. Several of these have been purified and characterized from a sugar-beet pulp isolate (Sakamoto et al. 2011, 2012, 2013a, b). Besides all major glycosyl hydrolase classes, four genes encoding the polysaccharide monooxygenases of the GH61 family have also been identified in the *P. chrysogenum* genome (Gusakov and Sinitsyn 2012). This class was recently identified as crucial for cost-efficient biomass hydrolysis for biofuel production (Langston et al. 2011).

Some of this biochemical arsenal is also used in order to compete out other fungi. An antifungal chitosanase PgChP (Pc12g07820) produced by an isolate from dry-cured ham was described by Rodríguez-Martín et al. (2010). Under laboratory conditions, transcription of this gene was very low in low-penicillin-producing strains such as NRRL1951 and Wisconsin54-1255 and, quite unexpected, much higher in production strains (MA van den Berg, IS Snoek et al. unpublished results). This is opposite for enzymes related to plant pathogenesis and infection, which are downregulated in the high production strains (Jami et al. 2009b), as these functions became redundant under laboratory and industrial conditions.

10.2.4 Taxonomy

Several species of the genus *Penicillium* do produce penicillins (Frisvad et al. 2004): *P. chrysogenum*, *P. dipodomyis*, *P. flavigenum*, *P. griseofulvum*, and *P. nalgiovense*. Fleming (1929) identified his fungus originally as *P. rubrum* and later re-identified it as *P. notatum*. However, morphological, extrolite, and molecular studies re-identified not only Fleming's isolate but also the NRRL1951 isolate as well as the sequenced strain Wisconsin54-1255 as *P. rubens* strains (Houbraken et al. 2011). *P. chrysogenum* is a highly related species but retained as a separate clade due to significant interspecific inhibition (Henk et al. 2011) as well as two other clades of *Penicillium* (species A and B in Fig. 10.1).

10.2.5 Evolutionary Path

Production of β-lactam antibiotics is not limited to saprophytic fungi as *Acremonium*, *Aspergillus*, and *Penicillium*; also some dermatophytic fungi from the genus *Trichophyton* (Hammadi et al. 2007) can produce β-lactam structures and do contain the respective biosynthetic genes. As two of the three penicillin biosynthetic genes (*pcbAb* and *pcbC*) are from bacterial origin (see, e.g., van den Berg et al. 2008) and the clustering of genes is well preserved among both cephalosporin- and penicillin-producing fungi (van den Berg et al. 2007), it is most likely that a full β-lactam biosynthetic pathway was acquired from prokaryotes producing cephalosporin (Fig. 10.1). From this ancestral β-lactam-producing fungus, a long evolutionary tract was initiated. The so-called expandase activity was lost, leading to a divergent class of fungi only producing penicillins. Next, a fungal gene encoding acyltransferase activity was "recruited" by the penicillin machinery, enabling more efficient penicillin production via hydrophobic side chains. The gene was duplicated and physically linked to the other penicillin biosynthetic genes (Spröte et al. 2008). Furthermore, the copied gene—*penDE* in *P.* chrysogenum—acquired via evolution a C-terminal targeting signal sorting the enzyme to the peroxisome where also CoA ligases are present (van den Berg et al. 2008; Kiel et al. 2009) that activate natural side chains enabling efficient production (Fig. 10.2). Further selection pressure made the original acyltransferase copy in Penicillia redundant and inactive, while in Aspergilli, it remained active (Spröte et al. 2008; García-Estrada et al. 2009; van den Berg et al. 2010). Interestingly, comparable evolutionary events took place in *P. brevicompactum*, producer of mycophenolate (MPA). One of the genes now present in the gene cluster, IMP dehydrogenase mediating MPA resistance, was duplicated before it was recruited to the MPA gene cluster. In this case, this duplication event took place before *P. chrysogenum* diverged from *P. brevicompactum*, but also before the MPA cluster was acquired (Hansen et al. 2012). The enzymes of *P. chrysogenum* have no clear function and are not very active.

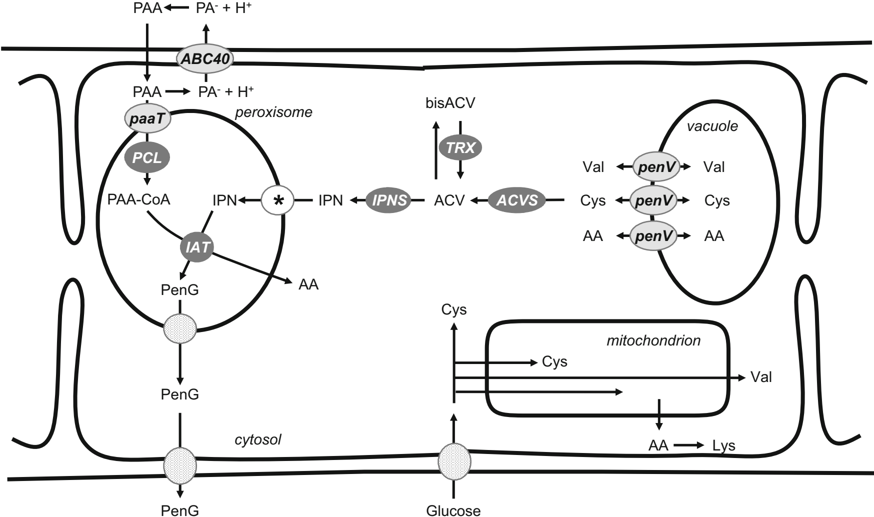

Fig. 10.2 Schematic overview of penicillin biosynthesis by *P. chrysogenum*. *Cys* cysteine, *AA* aminoadipate, *Lys* lysine, *Val* valine, *ACV* L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine, *bisACV* oxidized dimer of ACV, *IPN* isopenicillin *N*, *PAA* phenylacetic acid, *PA* phenyl acetate, *PAA-CoA* phenylacetyl-Coenzyme A, *PenG* penicillin, *ACVS* ACV synthetase, *TRX* thioredoxin reductase (Cohen et al. 1994), *IPNS* IPN synthase, *PCL* PA-CoA ligase, *IAT* isopenicillin *N*: acetyltransferase, *penV* vacuolar amino acid MFS transporter (Pc22g22150; Fernández-Aguado et al. 2013a), *ABC40* ABC transporter for PA secretion (Pc22g17530; Weber et al. 2012a), *paaT* peroxisomal MFS transporter for PAA (Pc21g01300; Fernández-Aguado et al. 2013b), asterisk, peroxisomal MFS transporter for IPN (most likely Pc22g22150, homologue of cefP; Ullán et al. 2010)

With the isolation of strain NRRL1951 from an infected cantaloupe bought on the local market in Peoria (Raper et al. 1944), submerged cultivation of *P. chrysogenum* became possible, with concomitant increased penicillin production. The concurrent need for antibiotics due to WWII spurred the development of an industrial production process. Classical strain improvement programs [reviewed by Lein (1986)] by several multinationals lead to the parallel development of the current industrial production strains producing several dozens of grams per liter, a staggering 20.000-fold higher than Fleming's isolate. This enormous improvement has been facilitated by many adaptations in fungal metabolism [reviewed in van den Berg (2011)]. The most prominent changes are the following:

- Elimination of side-chain (phenylacetic acid, PAA) catabolism
- Increased level of biosynthetic enzymes (via amplification of the gene cluster)
- Increased carbon flux towards the amino acid building blocks (aminoadipate, cysteine, and valine)

10.3 Penicillin Biosynthesis

Genetic analysis of mutants producing significantly reduced levels has provided the initial information for understanding the basics of penicillin biosynthesis (Queener et al. 1978; Cantoral et al. 1993; Fierro et al. 1995). The *P. chrysogenum* genome sequence (van den Berg et al. 2008) allows a more directed approach towards full understanding. Still, after more than 80 years and its genome displayed, penicillin production is not fully understood and opportunities for further improvement are identified.

10.3.1 Biosynthetic Pathway

The biosynthesis of all naturally occurring penicillins and cephalosporins starts with the condensation of three amino acids (Fig. 10.2): α-aminoadipic acid (AAA), L-cysteine, and L-valine into the tripeptide L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV). A large, non-ribosomal peptide synthetase (NRPS) ACV synthetase (ACVS) catalyzes as a single multifunctional enzyme the activation, fusion, and—in the case of valine—the epimerization (Wu et al. 2012) of the three amino acids. The enzyme sometimes makes "mistakes" and incorporates other amino acids, or the reaction is aborted too early (van den Berg et al. 1999), which is considered to be a potential energy drain during industrial production (van Gulik et al. 2000).

Secondly, the first β-lactam molecule is formed after oxidation and ring closure of ACV by the enzyme isopenicillin *N* (IPN) synthase (IPNS). IPN contains the characteristic β-lactam ring as well as the five-membered thiazolidine ring. Both ACVS and IPNS are cytosolic enzymes. In contrast, the two final steps of penicillin biosynthesis are located in a specialized organelle, the peroxisome (Fig. 10.2; see also next section). Here, the AAA moiety is exchanged for a novel side chain: phenylacetic acid (PAA) for penicillinG (PenG) or phenoxyacetic acid (POA) for penicillinV (PenV). Isopenicillin *N*-acyltransferase (IAT) first hydrolyses IPN into 6-aminopenicillinic acid (6-APA) and AAA, followed by the coupling of the activated side chain (PAA-CoA or POA-CoA). This latter activation is done by the peroxisomal enzyme phenylacetyl-CoA ligase, PCL (Lamas-Maceiras et al. 2006).

10.3.2 Compartmentalization

Penicillin biosynthesis is highly compartmentalized (Fig. 10.2). The synthesis of the amino acid precursors follows classical biochemical pathways via cytosol and mitochondria [reviewed by Evers et al. (2004)], the surplus being stored for later use in the vacuole (Affenzeller and Kubicek 1991). L-cysteine can be produced via

two different pathways in β-lactam-producing fungi: the transsulfuration and the sulfhydrylation pathway (Pieniazek et al. 1973). Theoretically, the yield of penicillin on glucose is higher when L-cysteine is synthesized via the direct sulfhydrylation pathway (Jørgensen et al. 1995). Here, cysteine synthesis is initiated through direct acetylation of L-serine by the cytosolic O-acetyl-L-serine sulfhydrylase (OASS). *P. chrysogenum* mutants lacking the direct sulfhydrylation pathway are disturbed in growth, suggesting it has another role than penicillin biosynthesis (Evers et al. 2004). The transsulfuration pathway on the other hand is located in the cytosol, where the cysteine is directly available for the cytosolic ACVS and IPNS (van der Lende et al. 2002). Moreover, transcriptome analysis of various strains of the *Penicillium* lineage shows that Pc12g05420 (encoding OAHS) was more induced than Pc21g14890 (encoding OASS) in higher producing strains, confirming the hypothesis that the transsulfuration pathway provides the cysteine for β-lactam production. The surplus of amino acids is stored in the vacuole and transported back into the cytosol by a MFS transporter named PenV (Pc22g22150; Fernández-Aguado et al. 2013a). Intriguingly, the transcript level of this gene is throughout all strains and conditions rather low and constant (MA van den Berg, IS Snoek et al. unpublished results), suggesting that the enzyme is always present at a basal level making sure that there is no limitation in the availability of amino acids.

Because of the compartmentalization, both IPN and the PAA side chain have to be transported into peroxisomes for further processing. Currently, it is still unclear how IPN and the final penicillin product(s) are transported across the peroxisomal membrane. Overexpression of pex11 led to a massive proliferation of peroxisomes (Fig. 10.3c, d) with a concurrent twofold increase in penicillin titers, while the levels of IAT and PCL remained constant (Kiel et al. 2005), suggesting that transport over the membrane was limiting penicillin synthesis. Two MFS transporters are involved in the translocation of biosynthetic intermediates essential for cephalosporin production by *Acremonium* over the peroxisomal membrane (Teijeira et al. 2009; Ullán et al. 2010). The closest *P. chrysogenum* homologue of the penN transporter cefM, Pc21g09220, is highly induced under penicillin-producing conditions in all strains tested (MA van den Berg, IS Snoek et al. unpublished results), suggesting its protein product has a role in penicillin biosynthesis. Unfortunately, the putative proteins from both species have no clear peroxisomal targeting sequence, and therefore, no solid conclusions can be drawn. IPN import into the peroxisomes is done by cefP; the closest *P. chrysogenum* homologue, Pc22g22150, is constitutively transcribed at low levels independent of penicillin production (MA van den Berg, IS Snoek et al. unpublished results).

A third MFS transporter, Pc21g01300, is off under nonproducing conditions and has a high transcript level under producing conditions with PAA in the medium (van den Berg et al. 2008). The enzyme encoded by the gene, aka paaT (Fernández-Aguado et al. 2013b), is located in the peroxisomal membrane and essential for import of PAA into the peroxisome.

The last barrier to be tackled is the cell membrane; the side-chain precursor has to be imported and penicillin has to be exported. PAA is imported by passive diffusion (Hillenga et al. 1995), but being a weak acid, it causes acidification of the

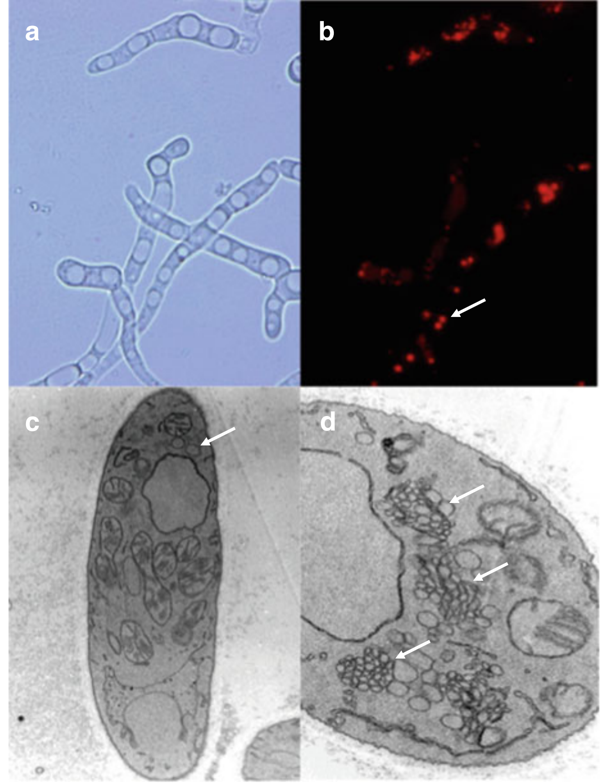

Fig. 10.3 Peroxisomes in *P*. *chrysogenum*. (**a**, **b**) High-producing strain DS17690 with DsRed-SKL under the control of P*pcbC* (Kiel et al. 2009). (**c**) Wisconsin54-1255; D, Wisconsin54-1255 with Pex11 overexpressed under the control of P*pcbC* (Kiel et al. 2005). Peroxisomes are indicated by the *arrows*

cytosol, and at high concentrations, it can become toxic to the cell (White et al. 1999). Therefore, while being imported, it is at the same time actively excreted by an ATP-binding cassette transporter (Pc22g17530 or ABC40; Weber et al. 2012a), leading to a futile cycle dissipating energy (Douma et al. 2012). The export of penicillin is dependent on ATP (van den Berg et al. 2001) and therefore most likely mediated by an ABC transporter, similarly to *A. nidulans* (Andrade et al. 2000). However, the closest homologue to this *A. nidulans atrD* is Pc20g01220, which is downregulated under (higher) penicillin-production conditions and therefore not the most likely candidate (MA van den Berg, IS Snoek et al. unpublished results). Several studies have attempted to identify the penicillin exporter through comparative and knockout studies but so far without any result (van den Berg et al. 2001, 2008; Weber et al. 2012c).

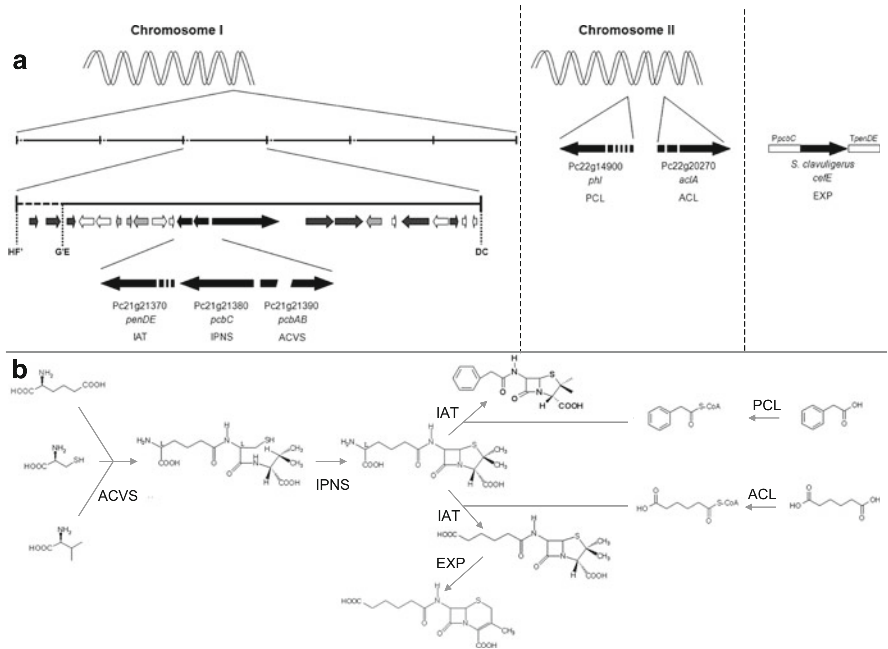

Fig. 10.4 Genome organization and metabolic pathway of β-lactam biosynthesis. (**a**) Genomic organization. The penicillin cluster is located on chromosome I and, as part of a larger region, amplified as tandem repeats in high-producing strains. The genes encoding the CoA ligases for the PAA and adipate side-chain precursors are both located on chromosome II as one gene copy. The expression construct encoding expandase, needed to produce adipoyl-7ADCA, under control of the *pcbC* promoter and *penDE* terminator, is integrated randomly in the genome. (**b**) Metabolic pathway. The three amino acid precursor aminoadipate, cysteine, and valine are fused together to form L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) by ACVS. IPNS catalyzes the oxidative ring closure to form isopenicillin *N*. The PAA and adipate side-chain precursors are activated by their respective CoA ligases, PCL (Lamas-Maceiras et al. 2006) and ACL (Koetsier et al. 2010). IAT catalyzes the exchange of the aminoadipate module with the activated side chain to form penicillinG and adipoyl-6APA, respectively. Expandase (EXP) transforms the latter molecule into adipoyl-7ADCA

10.3.3 Penicillin Gene Cluster

Production of secondary metabolites is highly regulated, often by means of a clustered gene set, including specific transcription factors and/or transporter(s). And while the genes (*pcbAB*, *pcbC*, and *penDE*) encoding the three penicillin biosynthetic enzymes (ACVS, IPNS, and IAT, respectively) are clustered on the genome (Fierro et al. 1995), neither specific regulators nor transporter genes can be identified (van den Berg et al. 2007) (Fig. 10.4).

Although *pcbAB* and *pcbC* are recruited via horizontal gene transfer from prokaryotes, the penicillin genes are positively controlled via a range of general

filamentous fungal transcriptional regulatory mechanisms [summarized in van den Berg et al. (2008)] including pH control (through PacC), carbon derepression (through the CCAAT-complex), nitrogen regulation (through Nre), and chromatin remodeling (through LaeA/VeA). Although no specific regulator was identified in the cluster, two specific transcriptional activators are known: Pta1 (Kosalková et al. 2007) and Rfx1 (Domínguez-Santos et al. 2012). Carbon catabolite repression is mediated by CreA and can be relieved by deleting the binding sites in the biosynthetic gene promoters (van den Berg 2011). Deletion of the *creA* gene led to unstable or cripple isolates, suggesting an essential role for CreA next to its role in controlling penicillin biosynthesis (van den Berg 2011).

10.3.4 Genome Insights

The 32 Mb genome of *P. chrysogenum* (van den Berg et al. 2008) revealed two remarkable secrets. First and most surprising, several ORFs encoding a range of putative β-lactam modifying enzymes were identified [see supplementary Table 6 in van den Berg et al. (2008)]. Laboratory *P. chrysogenum* strains cannot produce any cephalosporin and are dependent on the introduction of functional variants of IPN epimerase, expandase/hydroxylase, and deacetylcephalosporin C acetyltransferase in order to produce deacetylcephalosporin C and cephalosporin C (Ullán et al. 2008). Therefore, the identification of an isopenicillin *N* epimerase homologue (Pc12g11540) is unexpected, especially while it is actively transcribed and increased in higher producing strains (van den Berg et al. 2008). It is tempting to speculate this being a remnant of the original cephalosporin pathway in the ancestral fungus (Fig. 10.1), but this cannot be substantiated. Preliminary deletion studies suggest a minor role in penicillin biosynthesis (van den Berg et al. 2008); possibly it encodes an active competing enzyme. Other competing enzymes could be encoded by one or more of the hypothetical β-lactamases (3) or cephalosporin esterases (3). The presence of cephalosporin esterases hydrolyzing β-lactams has been reported counterintuitively for the cephalosporin producer *A. chrysogenum* (Velasco et al. 2001). Although *P. chrysogenum* does not produce cephalosporin C, these esterases are rather promiscuous and can hydrolyze a range of substrates. The transcript level of all six *P. chrysogenum* genes is rather low (van den Berg et al. 2008). Still, one of them (Pc12g13400, a cephalosporin esterase) is translated into protein, as it was identified during proteome analyses (Jami et al. 2009a). A mutation was introduced by classical strain improvement in a second putative cephalosporin esterase gene which will have hampered its function (van den Berg 2010). Ongoing functional studies have to resolve their role in β-lactam biosynthesis.

Secondly, comparative DNA analyses of different strains revealed changes in copy numbers of large clusters of genes. Six considerably large regions with different copy numbers between the sequenced strain and a high-producing strain were found [see supplementary Table 19 in van den Berg et al. (2008)]. As for the penicillin gene cluster, most of these regions are flanked by one or more

transposable elements, which may have played a role in the duplication or deletion of DNA. Two gene clusters with over 50 hypothetical genes, which were mostly low or not transcribed, are completely deleted from the genome of DS17690, the high-producing strain (van den Berg et al. 2008). Three clusters of genes have been amplified, including the penicillin biosynthetic gene cluster. The latter is present in 6–7 copies in DS17690.

10.4 Industrial Production

Classical strain and process improvements have led to a staggering annual β-lactam production of 65,000 metric tons (Lowe 2001) in large fermenters of 100–200 m^3. Moreover, metabolic engineering is applied to develop a direct fermentation route for cephalosporins (Cantwell et al. 1990), which is since the early 2000s performed at industrial scale at DSM in Delft, the Netherlands (Xie et al. 2001).

10.4.1 Classical Strain Improvement

The isolation of the NRRL1951 strain, which could produce penicillin during submerged fermentation (Raper et al. 1944), was the start of an unprecedented sequence of strain (and process) improvement leading to the current, highly optimized industrial *P. chrysogenum* strains (Fig. 10.1). Mutagens like UV-radiation, X-ray, or DNA-alkylating agents introduced the random DNA variations necessary to isolate improved mutants. Next to increased titers, one of the first targets was the reduction of the yellow chrysogenin pigment (Backus and Stauffer 1955). The later derived Wisconsin54-1255 strain isolated at the University of Wisconsin became the world laboratory standard (Elander 2002). This strain is the basis of most industrial strain lineages, and its DNA was used to determine the genome sequence for *P. chrysogenum* (van den Berg et al. 2008).

Besides the reduction in chrysogenin production, the many rounds of mutagenesis introduced a vast array of modifications in the genome; however, only two direct gene mutation-penicillin phenotype relations are known:

- pahA: The homogentisate pathway (phenylacetic acid catabolism) is largely inactivated in Wisconsin54-1255 and derived strains (Rodríguez-Sáiz et al. 2001), leading to an increased availability of side chain and thus increase in penicillin.
- *pcbAB-pcbC-penDE*: The penicillin gene cluster is amplified several times in production strains (Fierro et al. 1995; van den Berg et al. 2007).

A few other seemingly relevant mutations are known, for which the annotation or the physical location suggest a correlation with penicillin synthesis or secretion, but the direct relation with β-lactam biosynthesis was not shown:

- *erg25*: Encoding C4-setrol methyl oxidase, of which the orientation was reverted in Wisconsin54-1255 on the outer edge of the amplified region in production strains (van den Berg et al. 2007), possibly effecting the ergosterol content of the membranes.
- Pc12g04030: Encoding a putative cephalosporin esterase, of which the ORF was disturbed due to a 14 bp repeat, possibly decreasing β-lactam hydrolysis (van den Berg 2010).
- Pc12g00440: encoding a ABC transporter, which acquired a mutation during strain improvement, suggesting an effect in β-lactam transport (van den Berg 2010).

Some other obvious modifications to the fungal metabolism do contribute significantly to the improved penicillin titers, but the underlying mutations are not yet identified:

- Enhanced penicillin production rates correlate with increasing peroxisomal volumes (van den Berg et al. 2008; Meijer et al. 2010); this seems associated by the increase in transcript levels of 27 genes encoding peroxisome associated proteins (van den Berg et al. 2008).
- Expression of virulence factors, useful for natural isolates, is decreased in higher producing strains (Barreiro et al. 2012).
- NADPH generation is increased in higher producing strains (Barreiro et al. 2012).
- Transcript levels of amino acid biosynthesis genes and amino acid levels are increased in higher producing strains (van den Berg et al. 2008; van den Berg 2011).
- Morphological changes are associated with high-producing strains (Pócsi et al. 2007).
- High penicillin producer strains seem to have lost the ability to utilize cellulose, sorbitol, and other carbon sources (Jami et al. 2009b).

10.4.2 Genomics-Based Strain Improvement

The availability of the genome sequence stimulated the application of "omics" analyses in order to identify new leads for further improvement of the penicillin titers. The leads can be classified as follows: increase flux through primary metabolism, increase flux through biosynthetic pathway, improve energy balance, decrease side-chain catabolism, and improve organelle homeostasis (Table 10.3). The latter class is specifically oriented at the peroxisome, which after the recruitment of IAT became a very important compartment for high penicillin production in *P. chrysogenum*. Meijer et al. (2010) showed that there is a direct correlation between the number of peroxisomes and penicillin titer, which was extrapolated by either increasing peroxisome proliferation (Kiel et al. 2005) or preventing peroxisome autophagy by the vacuole (Bartoszewska et al. 2011), both modifications

Table 10.3 Gene modification leading to increased penicillin production (rates) in *P. chrysogenum*

Modification	Enzyme	Gene	Fold improvement	References
Deletion	Serine-threonine kinase[a]	Pc18g03490	1,37[b]	Bartoszewska et al. (2011)
	Acyl-oxidase[c]	Pc20g01800	1,6[d]	Veiga et al. (2012b)
	Acyl-CoA dehydrogenase[c]	Pc20g07920	3,7[d]	Veiga et al. (2012b)
	Oxalate hydrolase[e]	Pc22g24830	1,36[d]	Gombert et al. (2011)
	L-aminoadipate-semialdehyde dehydrogenase[f]	Pc22g06310	2[g]	Casqueiro et al. (1999)
Overexpression	IAT	Pc21g21370	1,6[b]	Weber et al. (2012b)
	PEX11[h]	Pc12g09400	2[b]	Kiel et al. (2005)
	pptA	Pc13g04050	1,3[g]	García-Estrada et al. (2008)
	PCL	Pc22g14900	1,35[g]	Lamas-Maceiras et al. (2006)

[a]Involved in autophagy (peroxisome sequestration by vacuoles)
[b]Based on penicillinV titer
[c]Involved in β-oxidation
[d]Based on adipoyl-6APA production rate
[e]Formation of oxalate (precipitates with calcium)
[f]lysine and amino acetate pathway
[g]Based on penicillinG titer
[h]Peroxisome proliferation factor

leading to a further increase in peroxisome number and penicillin titer. This latter finding is corroborated by the opposite experiment by Xu et al. (2012) where deletion of pcvA (Pc21g07250), encoding Rab GTPase, increased vesicle-vacuolar fusion and result in a decrease of penicillin titer.

10.4.3 Other β-Lactam Molecules

Classically, *P. chrysogenum* is used for producing penicillins, like penicillinV and penicillinG. The latter molecule was used for chemical modification towards the cephalosporin intermediate 7-aminodesacetoxycephalosporanic acid (7-ADCA, see Skatrud 1992), which is used a building block for semisynthetic cephalosporins such as cephalexin and cefadroxil. With the identification of the expandase enzyme in Streptomycetes and the subsequent introduction of the *cefE* gene in P. chrysogenum, an adipoylated version of the 7-ADCA molecule could be fermented (Cantwell et al. 1990; Skatrud 1992; Robin et al 2003). The adipoyl side chain can be easily removed by a specific acylase (Sio et al. 2002), and specific

side chains can be coupled either chemically or enzymatically in order to form the active pharmaceutical ingredient (API). Further fermentative modification of the adipoyl 7-ADCA into hydroxylated, acetylated, and/or carbamoylated versions is also possible in order to reduce even more additional chemical steps towards API's as cefuroxime (Harris et al. 2009b).

10.5 Conclusion

Eighty years after Fleming's discovery, penicillin production by *P. chrysogenum* is still the basis of one of the world's leading antibiotics. The genome sequence has shed new light on questions like why fungi are producing β-lactam antibiotics and why this particular fungus has become such a good producer. Continuous mutation and selection, both in nature and in the laboratory, has changed the blueprint of *P. chrysogenum* into a very efficient machinery, wherein several genes and enzymes needed to survive in nature have become obsolete and dysfunctional. Interestingly, some of these functions (i.e., synthesis of other secondary metabolites) are just suppressed by the penicillin production; when production is halted, the repression on these genes is relieved and synthesis might start. With respect to penicillin biosynthesis, new functionalities have been acquired, adapted, and duplicated in order to sustain the high flux from carbon source to the final product in the industrial production strains.

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Chapter 11
Rhizopus oryzae: Genetic Secrets of an Emerging Human Pathogen

Brian L. Wickes

11.1 Introduction

Rhizopus oryzae, sometimes referred to as a synonym of *Rhizopus arrhizus*, is a filamentous fungus that grows exclusively as a mold and is the most frequent cause of human mucormycosis (Ibrahim et al. 2012). Agents of mucormycosis include members of the family Mucoraceae (sometimes referred to as "pin molds" after the pin-like appearance of the sporangiophore and sporangia) and include fungi in the genus *Rhizopus*, *Absidia*, *Rhizomucor*, and *Apophysomyces*. Fungi in these genera, as well as the other families in the Mucorales order (Cunninghamellaceae, Mortierellaceae, Saksenaceae, Syncephalastraceae, Thamnidiaceae) combined with the order Entomophthorales, comprise a large group of fungi formerly classified as the zygomycetes (derived from the phylum Zygomycota). Clinical infections caused by these fungi are typically described as zygomycotic infections or zygomycosis, which are general terms suggestive of an infection caused by a member of the Mucorales or Entomophthorales (Kwon-Chung and Bennett 1992). However, there have been recent taxonomic changes to the phylum Zygomycota, which have led to a reclassification of the fungi in this phylum. Unfortunately, these changes have complicated the clinical study of *Rhizopus oryzae* and other members of the Zygomycota because the field of medical mycology encompasses both mycologists and clinicians, for whom taxonomy has a slightly different function. Clinicians typically must have a basic knowledge of all infectious diseases. Although many do specialize in medical mycology, it is difficult for clinicians to keep up with changes in taxonomy, particularly in the molecular era where revision of taxonomy often occurs as new technologies are applied to a given field. Basic researchers, on the other hand, need a naming system that is always current and

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_11,
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clearly categorizes the similarities and differences between members of the fungal kingdom due to a need to understand relationships among organisms. Consequently, with regard to this chapter, although the current taxonomy will be covered, the older classifications and names will be employed (Zygomycota, zygomycetes, Mucorales, etc.).

R. oryzae is arguably one of the best models of the pathogenic zygomycetes. This fungus causes ~70 % of the infections in humans (Roden et al. 2005) and exhibits typical zygomycetous phenotypes that include rapid growth rates, aseptate hyphae, wide distribution, and a saprobic lifestyle. These fungi are found throughout the world and are often associated with decaying vegetable matter including food, such as fruit, nuts, and cereal grains (Ribes et al. 2000). It is also one of the few zygomycetes in which a genome sequence has been completed and annotated (Ma et al. 2009). A transformation system exists and there are animal models of infection. Consequently, understanding *R. oryzae* at the genetic and molecular levels is an important strategy for understanding other zygomycetes, many of which are becoming increasingly important as pathogens.

11.2 Phylogeny

The phylogeny of *R. oryzae* has recently undergone some modifications. In medical mycology, new species are constantly discovered and changes in phylogenetic classification and nomenclature regularly occur. However, a recent driver of phylogeny has been the application of molecular biology to issues of phylogeny, taxonomy, and nomenclature. In many instances, molecular methods of phylogeny are quantitative and allow firmer species boundaries to be established, versus the descriptive nature of morphology, which can often be variable or insensitive. In fact, the phylum Zygomycota has undergone a large phylogenetic change in addition to the changes at the species level of *R. oryzae*.

11.2.1 Description of the Species

The names *R. oryzae* and its synonym *R. arrhizus* continue to be used interchangeably, including the research literature published as recently as 2012. This disagreement is not easily resolved as the discrepancy arises from differences in how to apply the rules of fungal nomenclature. *R. oryzae* was first described by Went and Geerling in 1895, while *R. arrhizus* was first described 3 years earlier by Fischer (Kwon-Chung and Bennett 1992). The *arrhizus* species epithet, therefore, has preference over the *oryzae* species epithet. However, there is some evidence that the original *R. arrhizus* isolate was an atypical form of *R. oryzae* (Scholer et al. 1983); thus, it was argued that *R. oryzae* is the proper name (Ellis 1998; Schipper 1984). This discrepancy is illustrative of the problems in fungal

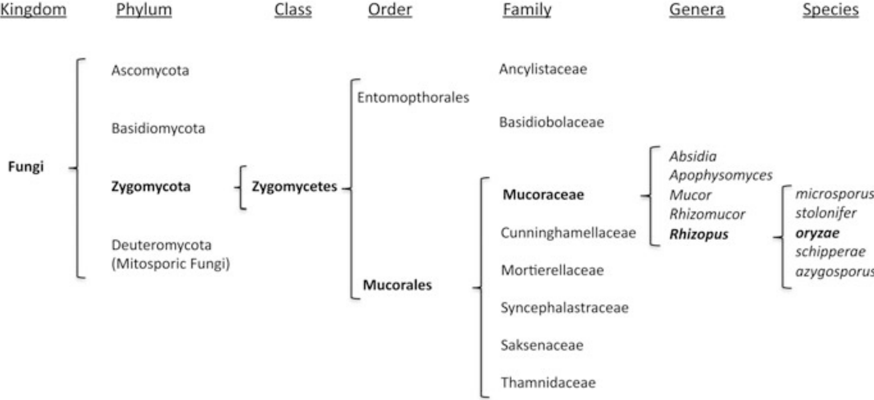

Fig. 11.1 *Rhizopus oryzae* taxonomy. *R. oryzae* is a zygomycetes found in the phylum Zygomycota. Importantly, the class Mucorales contains many, but not all, of the human pathogenic zygomycetes. The *Rhizopus* genus contains five pathogenic members, although other pathogenic species may be rarely isolated. *R. microsporus* can be further subdivided into three varieties, *oligosporus*, *microsporus*, and *rhizopodiformis*. Adapted from Ribes et al. (2000)

nomenclature, which adversely impact medical mycology. Unfortunately, resolution of the discrepancy would require material from the original isolate, which is not available. Because this chapter is research oriented, *R. oryzae* will be used as the species name, which also is the name attached to the isolate from which the genome sequence was derived. This sequenced isolate will be the strain used for any molecular references (genome information, genes, etc.) in this chapter as well.

The genus *Rhizopus* consists of approximately 8–13 species (Abe et al. 2006, 2010), depending on classification method (morphological vs. molecular) with five species being clinically significant: *R. oryzae*, *R. microsporus*, *R. schipperae*, *R. azygosporus*, and *R. stolonifer*. *R. microsporus* is further subdivided into three varieties (var. *microsporus*, var. *oligosporus*, var. *rhizopodiformis*) (Fig. 11.1). These species can be difficult to routinely discriminate in a clinical microbiology laboratory due to the rarity of isolation and, consequently, unfamiliarity with distinguishing features. However, molecular methods such as sequence-based identification can be straightforward in separating the five species. For the purposes of discriminating the different species of *Rhizopus*, the actin (act-1) and translation elongation factor 1 alpha (EF1α) genes have the most unique species GenBank entries. Sequences derived from these genes can be searched using the BLASTn algorithm at the NCBI GenBank website http://blast.ncbi.nlm.nih.gov/Blast.cgi. The three *R. microsporus* varieties, on the other hand, cannot be reliably discriminated based on sequence analysis or other molecular methods and can only be distinguished morphologically by the most experienced mycologists. Pathological specimens cannot be accurately identified to either the genus or species level; instead, they are generally just diagnosed as a zygomycotic infection.

11.2.2 Rhizopus oryzae *vs.* Rhizopus delemar

The genus *Rhizopus* is subdivided into three groups, the *stolonifer* group, the *oryzae* group, and the *microsporus* group, with the groupings based on morphologic characteristics such as size of the sporangia and sporangiophore, and rhizoid branching (Schipper and Stalpers 1984). Current methods utilizing sequence-based classification to establish phylogenetic relationships continue to support the initial morphologic grouping scheme (Abe et al. 2006). Subsequent phylogenetic investigations of *R. oryzae* arose, in part, due to its commercial importance as an organic acid producer. Use of *R. oryzae* in silage production requires strains that produce lactic acid; consequently, it is economical to use a strain that produces the optimal amount under the appropriate conditions. Variation in lactic acid production, specifically the existence of *R. oryzae* strains that either could or could not produce lactic acid, was already known (Oda et al. 2002). In an effort to efficiently screen strains for lactic acid producers, Abe et al. found a molecular method that utilized the rDNA sequence of the ITS region to devise a simple PCR-based method for identifying lactic acid-producing strains (Abe et al. 2003). This finding was extended by analyzing the sequences and gene organization of two genes required for lactic acid production, *ldhA* and *ldhB*, which had previously been cloned and characterized by Skory (2000). It was found that *R. oryzae* strains that possessed both an *ldhA* and *ldhB* gene formed one group within this species, which was called the lactic acid group, while strains that contained only the *ldhB* gene clustered as a separate group and were able to produce fumaric and malic acid, but not lactic acid (Oda et al. 2003; Saito et al. 2004). This group was called the fumaric–malic acid group.

Strong evidence for organic acid production serving as a basis for taxonomic discrimination was provided by Abe et al. (2007) who used extensive sequence analysis of multiple loci, as well as a genome sampling method called Amplified-Fragment Length Polymorphism (AFLP), to analyze these two groups to determine if the phenotypic differences had a phylogenetic basis. In this study, the sequences of a region within the ribosomal genes (ITS region), actin (act-1), translation elongation factor alpha (EF-1α), and the lactate dehydrogenase B gene (*ldhB*), as well as AFLP, were used to test the two organic acid clusters to determine if the same clustering was observed using molecular methods. The sample set was chosen from an earlier report using morphology for classifying the genus, which was the basis for the current *Rhizopus* classification scheme (Schipper and Stalpers 1984). The results of the molecular studies supported dividing the two organic acid clusters into distinct species. The cluster that produced fumaric and malic acid (FMA) was proposed to be renamed *Rhizopus delemar* (*Rhizopus delemar* (Boidin) Wehmer & Hanzawa), and the cluster that produced lactic acid (LA) was proposed to retain the *Rhizopus oryzae* name (*Rhizopus oryzae* Went & Prinsen Geerligs). Importantly, in addition to the molecular and phenotypic data being consistent with this classification, in the original study that was used to form the three groups, Schipper and Stalpers observed that mating studies of the FMA and LA groups revealed that only

strains originally classified in the LA group were fertile, which provided genetic evidence for the two observed clusters (Schipper and Stalpers 1984). In spite of some disagreement and lack of common use in clinical disciplines, there is strong support for the *R. delemar*–*R. oryzae* distinction.

11.3 Mycology

Medical interest in *R. oryzae* has recently been increasing due to the more frequent identification of the zygomycetes from patients. However, in spite of the importance of *R. oryzae* and other zygomycetes, the field has not reached critical mass in terms of investigators making new observations about these fungi. They can be harder to identify than other fungi and their overall rarity in clinical specimens means microbiologists are less familiar with them when they do arrive in the laboratory. However, completed genome sequences will encourage new investigations into *R. oryzae* and other zygomycetes, which should yield both commercial and medical benefits.

11.3.1 Growth and Identification

Morphologically, *R. oryzae* is typical of many of the zygomycetes in that it exhibits extremely rapid growth rates, quickly spreading horizontally and vertically on a petri dish, often reaching the side borders and the lid within a matter of days. If grown on slants, hyphae can fill the whole tube (Fig. 11.2). There is abundant growth at 25–37 °C, good growth at 40 °C, and no growth at 45 °C. Younger cultures are whitish but become brownish gray to darker gray with a wooly texture. Mature sporangia can appear as black spots on the colony and tend to be on the edges or in the center. Sporangiophores can reach a length of 1,500 μm long × 18 μm wide and may be simple or branched, smooth-walled, and nonseptate (coenocytic) and are opposite rhizoids that are usually produced in groups of three or more (Fig. 11.3) (Ellis 1998). Sporangia are grayish black, globose, with diameters of up to 175 μm, and contain sporangiospores that are usually rhomboid or lemon-shaped and striated (Kwon-Chung and Bennett 1992). Most isolates will grow on standard mycological media such as potato dextrose or Sabouraud's, or a defined glucose-salts basal medium, depending on what is being studied. Identification of *R. oryzae* and other members of the genus is done mainly through morphology, although other methods, which are based primarily on DNA sequence of a variety of genes, is gaining prominence (Balajee et al. 2009). In fact, sequence-based diagnostic methods can often discriminate at the species and sometimes the subspecies (i.e., varietal) level, depending on the target locus or loci.

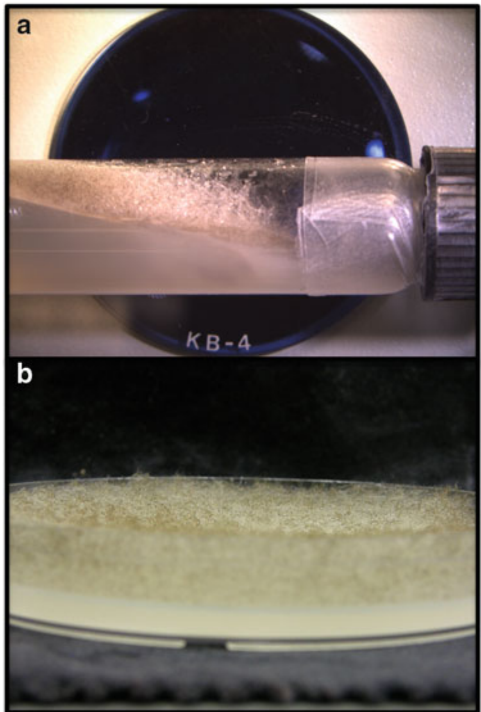

Fig. 11.2 *Rhizopus oryzae* growth rate. Growth rate of *R*. *oryzae*. (**a**) Growth on a 16 × 100 mm screw cap potato dextrose agar slant incubated at 37 °C for 72 h. Slant was photographed with an Olympus SZX12 microscope at ×5 power. (**b**) Growth on a 100 × 15 mm potato dextrose agar petri dish incubated at 37 °C for 72 h. Lids were elevated above the lip of the dish and then fixed in place with tape. The lid was then sealed with parafilm that was punctured to allow air circulation. Note sporangiophores that have grown above the top edge of the petri dish. Plate was photographed with an Olympus SP-560UZ optical zoom camera

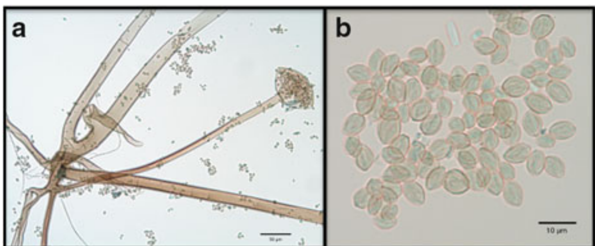

Fig. 11.3 *Rhizopus oryzae* microscopy. Microscopic slide cultures produced on potato flakes agar. (**a**) Slide showing a collapsed sporangium, sporangiospores, sporangiophore, and the rhizoids. Stolons are hyaline to *brown*. Sporangiophore is unbranched. (**b**) Slide showing sporangiospores, which are lemon-shaped and striated. Pictures courtesy of Deanna Sutton, Department of Pathology, Fungus Testing Laboratory, The University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

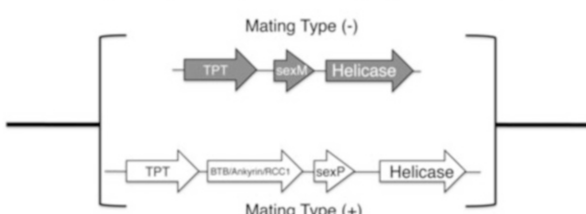

Fig. 11.4 *Rhizopus oryzae* mating locus. Organization of the *R. oryzae* mating-type locus showing genes found in the (−) and (+) mating type. The mating-type-specific information of either locus is shown within the *brackets*, which delineates the boundaries of the two loci. The *darker lines* on either side of the *brackets* show common regions of the mating-type chromosome, which flanks the locus

11.3.2 Sexual Cycle

R. oryzae is heterothallic with a bipolar mating system containing two mating types, (+) and (−) (Schipper 1984). Mating in *R. oryzae* is seldom observed under any conditions, and in spite of the production of zygospores from paired matings, germination is rare and the demonstration of recombinant progeny under laboratory conditions has been difficult (Gryganskyi et al. 2010). Consequently, the utility of a sexual phase as a research tool in *R. oryzae* has not been demonstrated. Crosses involving tester strains to confirm species identity are not useful in *R. oryzae* diagnostic studies because not all strains are fertile, and induction of the sexual state can occur across species (Schipper 1984; Gryganskyi et al. 2010). Nevertheless, Schipper was able to use this characteristic to assign mating types to multiple culture collection isolates by crossing a mating-type (−) *R. microsporus* strain with a number of *R. oryzae* strains to induce zygospore production and assign mating type, of which both (−) and (+) were observed. However, none of the zygospores were fertile in these crosses, demonstrating the difficulty of using the mating reaction as a molecular tool, such as for crossing out undesirable characteristics or for following inheritance of genetic markers.

The only molecular study of the *R. oryzae* mating-type locus was conducted by Grygansky et al. (2010). In their study of the *R. oryzae* complex, they used knowledge of the genes within the mating loci of *Phycomyces blakesleeanus* and *Mucor circinelloides* (Idnurm et al. 2008; Lee et al. 2008) to identify the genes within the *R. oryzae* locus and how they were organized. Consistent with earlier studies using tester strains, it was observed that only two molecular types were found within a collection of *R. oryzae* isolates, with each strain displaying an organization that allowed it to be classified as either (+) or (−) (Fig. 11.4). The (−) mating-type allele contained the *Phycomyces blakesleeanus* and *Mucor circinelloides* homologs, TPT (triose phosphate transporter), SexP, and an RNA helicase. The (+) allele was also syntenic with *Phycomyces blakesleeanus* and *Mucor circinelloides* and had homologs of TPT, SexP, and an RNA helicase. The

(+) allele, in addition, had a chimeric section containing three parts, BTB/Ankryn/RCC1, based on comparison to the *R. oryzae* genome sequence annotation. This region lies between the TPT and SexP genes; however, its function is unclear. In addition to a potential functional role, it may also serve to block recombination within the mating loci due to absence of homology with the (−) locus, thus contributing to keeping the locus intact. Similarly, the roles of the common helicase and TPT genes are unclear; however, one or both may insure the inheritance of the locus by being essential for viability.

Although obtaining recombinant progeny in the laboratory via the production of zygospores has been difficult to demonstrate in *R. oryzae*, it appears that mating may be an important part of the life cycle and might actually occur quite frequently. Gryganskyi et al. (2010) actually found zygospore production to be quite frequent in their sample collection, with sporogenesis occurring in about 33 % of their isolates. Schipper (1984) also found many *R. oryzae* isolates capable of producing zygospores when crossed with the appropriate tester, so obtaining viable progeny that are truly recombinant may require novel mating conditions in the laboratory or perhaps recovering isolates from a breeding population from a common niche. In fact, the recent description of mating in *Aspergillus fumigatus* reported mating reactions that initially took almost 6 months to produce cleistothecia and ascospores (O'Gorman et al. 2009), but when studies were undertaken to optimize conditions and search for a suitable tester pair, mating efficiency was increased greatly (Sugui et al. 2011). Consequently, further development of mating in *R. oryzae* may yet lead to a powerful and useful genetic tool for future studies.

11.4 Commercial Uses

Most of the early knowledge of *R. oryzae* came from food and commercial interest. In fact, while the medical importance of *R. oryzae* has been recognized for a little over 100 years, its role in food production is centuries old. Ironically, the basic research interest of *R. oryzae* focused mainly on strain characterization and strain improvement.

11.4.1 Tempe

Although *R. oryzae* is an important human fungal pathogen, perhaps its greatest impact on humans is via commercial products produced by the fungus. *R. oryzae* produces an extremely wide variety of commercially useful metabolites, including a number of enzymes, polymers, alcohols, esters, and organic acids (Ghosh and Ray 2011). Interestingly, this metabolic diversity has been confirmed by the number, type, and organization of genes found within the *R. oryzae* genome (Ma et al. 2009).

Among the oldest commercial use of *R. oryzae*, which dates back more than 1,000 years, is in the production of Tempe or Tempeh. Tempe is an important food source for some cultures and is produced by the fermentation of soybeans, which in contrast to fermented beverages, actually incorporates the fungus into the final product such that Tempe consists of the fermented beans bound together with fungal mycelia and flavored with fungal metabolites produced during the fermentation process. Just as for alcoholic beverages, discriminating species and strains of *Rhizopus* is crucial to the qualities of the final product, whether it is food or a *Rhizopus* metabolite. In fact, *R. microsporus var. oligosporus* may be the most common species of *Rhizopus* used to produce Tempe (Hachmeister and Fung 1993). Strain improvement and selection have moved Tempe production from the smaller home-based fermentations to industrial-level production (Steinkraus 1985; Nout and Kiers 2005). As a result, the increased commercial value has generated more interest in improving the fermentation process, which has driven more basic microbiological and genetic studies. These studies have provided a more mechanistic understanding of what genes are responsible for some of the metabolic characteristics that *R. oryzae* is known for (Meussen et al. 2012).

11.4.2 Enzymes

Some of the enzymes produced by *R. oryzae* and other species include cellulases (enzymes useful for biomass degradation, which function by hydrolyzing cellulosic plant material, including cell walls), pectinases (needed for fruit juice and wine processing), tannase (tannic acid decomposition), phytase (releases inositol from plant seeds), amylase (degrades starch), lipase (fat and oil degradation), protease (protein degradation, often used in the food, leather, tanning, and detergent industries), RNASE (ribonuclease, which degrades RNA), urease (urea degradation), and others (Ghosh and Ray 2011). The saprophytic lifestyle of *R. oryzae* undoubtedly played a role in the evolution of numerous enzymes that facilitated the breakdown and metabolism of plant biomass. Additionally, this nutritional versatility also likely has contributed to the success of *R. oryzae* as a pathogen. The production of numerous types of cellulases makes *R. oryzae* a potentially useful microbe in bioengineering since these enzymes can degrade cellulosic or agricultural wastes into products that can be used as fuel or chemicals, depending on downstream processing steps. In some cases, these processes can be coupled, such as during the production of lactic acid, which yields ethanol during the fermentation process (Zheng et al. 2009). Strain modification can be used to enhance or alter these processes in commercially useful ways (Bai et al. 2004).

11.4.3 Organic Acids

R. oryzae is able to produce a number of useful and important organic acids. Among them are lactic acid, fumaric acid, and malic acid. Ethanol can also be produced during this process since the pathways leading to the production of all four products start with pyruvate (Skory and Ibrahim 2007). Organic acid production from *R. oryzae* has been studied for almost 100 years (Ghosh and Ray 2011) with the type of acid produced (lactic or fumaric–malic) being useful for *R. oryzae* strain characterization (see above). While *R. oryzae* can produce other types of organic acids, the production of lactic acid and fumaric acid is the most important and the most studied.

Fumaric and malic acid are Krebs (or TCA) cycle intermediates that are derived from fermentative growth on high carbohydrate substrates to produce pyruvate, which can enter the Krebs cycle through conversion to citrate and then be metabolized to fumarate and malate. These acids are often used as "acidulants," which are food additives that function to lower pH and/or increase tartness (Meussen et al. 2012). Subsequent steps to produce fumarate from succinate, and then malate directly from fumarate, follow the traditional Krebs cycle pathway. However, in *R. oryzae*, experimental evidence suggests that these two organic acids are produced by an alternate pathway of the Krebs cycle starting from pyruvate and proceeding through oxaloacetate to malic acid and finally fumaric acid via the reductive branch of the Krebs cycle (Romano et al. 1967). As noted above (see Sect. 11.2), not all species of *R. oryzae* traditionally were able to produce fumaric acid, which led to an early interest in *R. oryzae* genetics. These studies ultimately identified the genetic basis that determines which organic acids were produced based on the presence or absence of lactate dehydrogenase. *R. delemar* lacks this enzyme and thus produces fumaric and malic acid (Skory and Ibrahim 2007; Skory 2004). The other group, which contains both *ldhA* and *ldhB*, retains the *R. oryzae* name and produces lactic acid from pyruvate.

Lactic acid, or more specifically, L-(+)-lactic acid, is used as a food or feed acidulant, but it can also be used for the production of plastics, solvents, cosmetics, food antimicrobials, or oxygenated chemicals (Ghosh and Ray 2011; Datta and Henry 2006). While bacteria are major sources of commercially produced lactic acid, *R. oryzae* has been increasingly used as a source of this compound, due in part to its ability to produce an optically pure form on defined growth media (Skory 2003). In addition to modifying growth conditions (i.e., temperature, aeration), strain modification, such as using mutagenesis or recombinant DNA methods, has led to increases in the amount of lactic acid that can be produced per gram of substrate (Bai et al. 2004; Skory 2004; Skory et al. 1998).

The focus on the genetics and molecular biology of *R. oryzae* was driven by the potential commercial applications of the fungus. However, this interest also facilitated new avenues of investigation into *R. oryzae*, which eventually led to a genome sequence project. Analysis of the genome sequence has yielded extensive

information related to commercial applications of *R. oryzae*. However, the main justification of sequencing this fungus arose from its role as the most frequent pathogen in mucormycosis.

11.5 Pathogenicity

In general, infections caused by the zygomycetes are rare compared to other medically important fungi. Within the phylum, genera within the order Mucorales are the most frequent cause of disease, with *R. oryzae* being the most common of all the zygomycetes (Roden et al. 2005). Importantly, zygomycotic infections are often referred to as mucormycosis or zygomycosis. Mucormycosis refers to pathogenic members of the Mucorales; however, there are other non-Mucorales genera that can be human pathogens. These non-Mucorales zygomycotic pathogens are typically members of the Entomophthorales, such as *Basidiobolus* sp., but are very rarely recovered. Nonetheless, the term zygomycosis is a catch-all term to indicate that the fungus is a member of the phylum Zygomycota. Mucormycosis simply reflects more specific taxonomic information in that the identity of the isolate has been narrowed down to the order Mucorales. From a clinical perspective, both terms are used and combined with the recent recommendations for changes in taxonomy due to the emergence of the *R. delemar* species epithet reflect the ongoing confusion of naming and categorizing these fungi.

11.5.1 Host Status

In spite of being a generally rare infection, mucormycosis is typically an aggressive one, reflecting, in part, the rapid growth rate of these fungi. The aggressive nature of these infections has lead to mortality rates of approximately 45–80 %, with underlying disease affecting the rate (Lanternier et al. 2012). When dissemination to other sites in the body occurs, mortality rates can approach 100 % (Eucker et al. 2001). Risk factors for infection by *R. oryzae* and for most zygomycotic agents in general include diabetes, certain types of cancers, immunosuppression, particularly in organ transplantation, diseases, and therapies that affect iron homeostasis, drug abuse, and trauma that includes traumatic implantation of environmental material (dirt, debris, plant material) (Ibrahim et al. 2012; Ribes et al. 2000; Lanternier et al. 2012; Eucker et al. 2001). AIDS in and of itself is not a risk factor for infection; however, underlying factors such as drug abuse or neutropenia have resulted in mucormycosis appearing in this patient population with increasing frequency (Van den Saffele and Boelaert 1996). In general, most patients that are infected with *R. oryzae* or other mucormycotic agents will have some underlying immunosuppressive condition that predisposes to opportunistic mycosis. The exception is trauma, which can lead to infection in two ways. In the first way,

although initially healthy, trauma patients can become susceptible to infection due to trauma-induced immunosuppression. An example would be burns, which result in substantial alteration of host stasis as well as allowing access to external pathogens due to breach of skin. Secondly, trauma can lead to skin breaches that allow aerosolized fungal spores to cross the skin barrier by entering a wound, or fungal elements in the form of spores and/or hyphae can cross the skin barrier by direct inoculation via impacted trauma debris that carries fungal elements. Spores can then germinate, or hyphal elements can colonize tissue. If the host response cannot control the colonizing fungus, tissue invasion can follow, which can lead to necrosis, angioinvasion, dissemination, and ultimately death.

11.5.2 Clinical Manifestations and Infection Sites

For *R. oryzae* infections, one of the most common risk factors is diabetes with accompanying ketoacidosis. The most common sites of infection tend to be the skin, lung, gastrointestinal tract, and paranasal sinus (Kwon-Chung and Bennett 1992; Hagensee et al. 1994; Record and Ginder 1976; Rammaert et al. 2012). Sinus infections that initially manifest or progress to rhinocerebral infections are overwhelmingly caused by *R. oryzae* (~90 %) (Scholer et al. 1983). Infections typically are caused by inhalation of spores, which can germinate in the sinus or reach the lung alveoli where they germinate, colonize, and then invade tissue, often with a tropism for blood vessels from which dissemination can occur, depending on the host status. Rhinocerebral infections progress rapidly and are life threatening due to proximity to the brain. These infections must be diagnosed quickly and are usually treated with extensive surgical debridement, antifungal therapy, and reversal of underlying immunosuppressive conditions if possible. However, even with aggressive treatment, rhinocerebral infections still usually have poor outcomes (Rinaldi 1989). Pulmonary infections can mimic infections caused by *Aspergillus* sp. and may need live culture or histopathology for proper identification due to nonspecific symptoms that can appear with imaging or other diagnostic methods (Fig. 11.5). Unfortunately, in the absence of live culture or molecular-based methods, species determination is almost impossible, and in many cases, obtaining specimens for live culture is not possible. Cutaneous infections can arise from the insertion of intravenous lines that carry contaminating fungal elements from the skin or from direct inoculation of fungal elements into the skin. The infection site can undergo necrosis with the formation of black eschar if the fungus successfully invades the surrounding tissue (Eucker et al. 2001). Gastrointestinal infections also can occur and probably reflect oral transmission due to consumption of contaminated material (Neame and Rayner 1960). However, the disease can occur in premature babies with or without skin lesions and obviously in the absence of any contaminated food consumption, suggesting an unknown origin (Zaoutis et al. 2007).

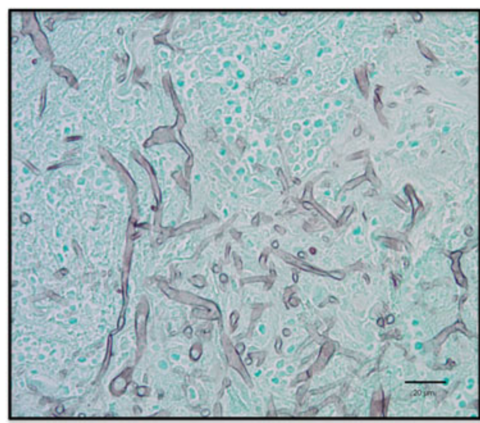

Fig. 11.5 *Rhizopus oryzae* histopathology. Gomori methenamine silver (GMS) stain of infected tissue. Hyphae are coenocytic, flattened, and ribbon-shaped with irregular branching

11.5.3 Virulence Factors

Factors that affect and enhance virulence in *R. oryzae* are coincident with the phenotypes most important for survival in the natural niche as *R. oryzae* infections are opportunistic in nature. *R. oryzae* is not a commensal of humans or animals, nor is it shed during infection; therefore, mammalian or other animal immune systems have not played a role in the evolution of genes that could make the organism pathogenic. Instead, the genes or phenotypes that *R. oryzae* displays that enhance virulence reflect the normal ecosystems in which *R. oryzae* lives. However, in spite of the saprophytic lifestyle of *R. oryzae*, a number of phenotypes have been identified that play a role in virulence, and in a few instances, the corresponding genes that influence these phenotypes have been identified. Unfortunately, the majority of what is known about the repertoire of virulence factors that *R. oryzae* possesses has been deduced from studies of other specific Mucorales. These are, therefore, possible virulence factors of *R. oryzae* suggested by taxonomic relationships, but their function remains to be proven.

The role of iron in fungal pathogenesis, including the major systemic fungal pathogens, is well known (Hilty et al. 2011; Haas 2012; Jung and Kronstad 2008; Chen et al. 2011). *R. oryzae* is no exception with regard to the importance of iron in virulence. Numerous studies of *R. oryzae* virulence have identified a major virulence factor, which involves how the fungus acquires iron in vivo and how host iron availability influences the probability of being infected by *R. oryzae*. Much of the host–pathogen relationship, however, seems to revolve around iron availability during *R. oryzae* infection. This dynamic makes iron acquisition, and how the host tries to deny access to iron, the most studied of the *R. oryzae* virulence factors (Nairz et al. 2010). Non-trauma *R. oryzae* infections often involve some aspect of iron availability, specifically levels of unbound iron in the serum that are accessible to the fungus (Howard 1999). Mammals use multiple methods to sequester iron, such as the carrier proteins ferritin, lactoferrin, and transferrin, which keep free iron away

from pathogens, while reducing potential toxicity. *R. oryzae* grows poorly in serum, unless exogenous iron is added (Artis et al. 1982). However, there is a second component to iron acquisition by *R. oryzae*, which involves pH. Because diabetes is a risk factor for mucormycosis, much of what is known about how *R. oryzae* uses iron in vivo comes from these patients. Diabetics who are in ketoacidosis due to their disease have elevated levels of iron in their serum. It is the combination of free iron and the acidotic state of these patients that makes iron available to the fungus since adding free iron to serum does not stimulate fungal growth unless the serum is acidic (7.3–6.88) (Artis et al. 1982). Furthermore, using diabetic mice, Ibrahim et al. provided further proof of this mechanism by showing that chelation of iron can be used therapeutically to keep iron away from *R. oryzae*, even in the conditions (diabetic ketoacidosis) that would make it available (Ibrahim et al. 2006; Ibrahim et al. 2007). In fact, *R. oryzae* is genetically primed to grab iron any way it can. Indeed, patients who are treated with deferoxamine, a bacterial siderophore, are at elevated risk for infection because *R. oryzae* recognizes this compound as a xenosiderophore, which will strip iron from transferrin and then, in turn, allow it to be bound by *R. oryzae* (Boelaert et al. 1993). Using traditional molecular methods for studying genes involved in pathogenesis, Ibrahim et al. demonstrated the role of *FTR1*, an *R. oryzae* permease, in iron uptake by disrupting the gene and using an anti-Ftr1 antibody to show that elimination of the Ftr1 function reduces virulence (Ibrahim et al. 2010). Finally, the genome sequence project has revealed the presence of heme oxygenase homologs, which may function in allowing the fungus to obtain iron from host hemoglobin, perhaps suggesting in part, the tropism for blood vessels during infection (Ibrahim et al. 2012; Ma et al. 2009).

In addition to obtaining iron from the host, another key phenotype that is almost always required for a pathogen to be virulent is the ability to adhere to host tissue. Adherence is generally going to precede invasion, and in the case of *R. oryzae* pathogenesis, invasion of tissue and blood vessels is a key component of tissue damage, which is a hallmark of infection. *R. oryzae* has been shown experimentally to adhere to, and damage, endothelial cells in a specific manner since adherence to nonliving tissue, such as plastic, is not observed (Ibrahim et al. 2005). The process requires direct contact but can be blocked by the addition of iron chelators, again, showing the importance of iron to pathogenesis (Ibrahim et al. 2005). Interestingly, there is some suggestion of the role of a toxin in this process. *R. oryzae* produces a toxin (agroclavin) and other members of the genus have also been shown to produce toxins (Ribes et al. 2000). Furthermore, endothelial killing can be brought about by dead cells, suggesting that this killing may be mediated by a factor that could be a toxin or toxin like (Ibrahim 2011).

Virtually all microbial pathogens need the ability to grow at host ambient temperature, which is 37 °C for humans. Although this temperature is a given for pathogens, most fungi cannot grow at this temperature, let alone deal with the multiple aspects of host defenses, both active and passive. *R. oryzae* grows well at this temperature and is, therefore, not inhibited in normal hosts nor by elevation of body temperature through fever. In fact, since most infections likely begin via introduction of spore aerosols, evidence of *R. oryzae* growing satisfactorily at this

temperature comes from animal models in which inocula are introduced as a spore suspension, which ultimately results in the hyphal morphology in tissue as the spores germinate and invade tissue (Ibrahim et al. 2010).

11.5.4 Treatment

Treatment of *R. oryzae* infection, as well as many mucormycotic infections, must consider the aggressive nature of these infections and how rapidly they can progress. Consequently, a strong index of suspicion is needed so that treatment is not delayed, something that is often not possible due to the large number of other factors, many more common, which could be mimicking symptoms of an *R. oryzae* infection. Two other factors are crucial to infection management, surgical debridement and antifungal therapy (Arnaiz-Garcia et al. 2009), although even with surgical intervention, mortality can be as high as 60 % (Rabie and Althaqafi 2012). Unfortunately, the Mucorales are a diverse collection of zygomycetes that display various phenotypes, including selective resistance to antifungals, further demonstrating the necessity of identifying these fungi correctly. Vitale et al. (2012) published an extensive study on antifungal susceptibility of the Mucorales. *R. oryzae* was resistant to terbinafine, 5-FC, caspofungin, micafungin, voriconazole, and fluconazole, but was sensitive to posaconazole, itraconazole (less susceptible than most zygomycetes), and amphotericin B (although somewhat reduced susceptibility). However, for disseminated infections, *R. oryzae* can be refractive to most antifungal agents.

11.6 Genome Sequence

The genome sequence for *R. oryzae* was completed at the Broad Institute http://www.broadinstitute.org/annotation/genome/rhizopus_oryzae/MultiHome.html and reported in 2009 (Ma et al. 2009).One of the major arguments for sequencing this fungus was that the sequence would represent the first sequence obtained from an early lineage of the fungal phylogenetic tree. Based on the distinguishing characteristics of *R. delemar* and *R. oryzae*, the isolate that was sequenced, 99–880, was actually phenotypically *R. delemar*. Nonetheless, a substantial amount of information was obtained from the annotation of the genome, including some of the genetic foundations for pathogenesis. The major findings of the genome sequence centered around evidence of a whole-genome duplication followed by subsequent duplications of selected gene families. Analysis of the genome focused on developing a better understanding of the genetic basis of pathogenesis.

11.6.1 Genome Annotation

Final assembly of the sequence data revealed 15 linkage groups, which was supported by combining sequence data with optical mapping data (Ma et al. 2009). Putative telomeric sequences were identified $(CCACAA)_n$ with the help of the optical maps. The total number of predicted protein coding genes was 17,467 with most genes having introns (avg 2.32 introns/gene) that averaged 79 bases in length (Ma et al. 2009). The two most significant observations from the assembled sequence were the existence within the genome of an enormous amount of repetitive sequences and evidence that the genome had undergone a whole-genome duplication.

The number of repetitive elements consumed more than 9 Mb of the genome (approximately 20 %) and was made up mostly of transposable elements and their remnants. The most abundant type of element was Ty3/gypsy like. The transposons could be divided into two classes: Class I, which were the retrotransposons, and Class II, which were the DNA transposons (Ma et al. 2009). Insertion sites showed a bias, with many localizing close to tRNA genes. Some of the elements were active as they were detected in expressed sequence tags.

The second major observation from the genome sequence assembly was the existence of an apparent genome duplication. Initial evidence that the genome was duplicated came from the detection of a paralog for almost half of the genes in the genome. The duplicated sequences cover the entire genome and consist of 648 gene pairs that appear in duplicated sections, which contain 3–9 duplicated genes per section (Ma et al. 2009). Much of the evidence for concluding that a genome duplication occurred was derived from the conservation of the order and orientation in which the paralogs appeared. After the genome was duplicated, genes were gradually lost, yielding the "modern" *R. oryzae* genome, which retained roughly 650 of the original duplicates. Importantly, the study by Ma et al. (2009) allowed these investigators to use the sequence to make certain inferences about the phenotypes that *R. oryzae* exhibits both as a saprophyte and a pathogen. These inferences related the observation of gene families that were created from the whole-genome duplication to the specific phenotypes that *R. oryzae* exhibits, which could be attributed to the genes that constitute each family.

11.6.2 Duplicated Protein Complexes

The lifestyle of *R. oryzae* is consistent with an organism that has an extensive genetic reserve to draw upon depending on the local environment. This flexibility is consistent with the commercial utility of the fungus in which its fermentative and degradative abilities have been exploited. Conversely, the organism can also be an aggressive pathogen that is characterized by rapid growth once an infection is established and the organism begins to invade tissue. In fact, the rapidly invasive

phenotype is a hallmark of *Rhizopus* infections. A crucial requirement for the nutritional flexibility and rapid growth of *R. oryzae* is the need to generate energy for these metabolic processes. The protein complexes that are duplicated in the genome fit this requirement and include proteins that constitute the electron transport chain, the V-ATPase complex, and the ubiquitin–proteasome systems (Ma et al. 2009). For each system, the core subunit is duplicated.

Electron transport proteins mediate the flow of electrons to oxygen while generating ATP during the process. The electron transport complexes that were predicted to be duplicated in *R. oryzae* include complex I (NADH–ubiquinone oxidoreductase), which accepts electrons from NADH; complex II (succinate dehydrogenase–CoQ oxoreductase), which accepts electrons from succinate; complex III (cytochrome reductase), which accepts electrons from coenzyme Q; and complex IV (cytochrome oxidase), which accepts electrons from cytochrome C. The duplication of these complexes may enhance the metabolic flexibility of *R. oryzae* while also contributing to the rapid growth rate of this fungus.

V-ATPases are proton pumps that require ATP and are found in intracellular compartments as well as the plasma membrane. They are important for acidification of intracellular compartments, membrane trafficking, protein degradation, and coupled transport (Forgac 1998). Individual V-ATPase components have not been studied in detail in *R. oryzae*; however, they have been studied in other fungi, including fungi pathogenic for humans. In *Histoplasma capsulatum*, the V-ATPase catalytic subunit A, *VMA1*, was found to be required for dimorphism, iron acquisition, and virulence (Hilty et al. 2008). Similarly, the *Cryptococcus neoformans VPH1* gene was found to negatively affect melanin production, capsule production, and growth at 37 °C, all of which are required for virulence (Erickson et al. 2001). Finally, the *Candida albicans VMA7* gene, a V-ATPase subunit, was shown by Polterman et al. (2005) to be required for vacuolar acidification, growth at alkaline pH, metal ion homeostasis, dimorphism, and virulence.

Most of what is known about the ubiquitin–proteasome pathway in pathogenic fungi comes from *Saccharomyces cerevisiae* and to a lesser degree *Candida albicans*. None of the individual components of this pathway have been studied in *R. oryzae*; however, homologs of other fungal proteasome subunits have been identified in the *R. oryzae* genome sequence including homologs of the *PRE*, *PUP*, *RPT*, and *RPN* proteins, which make up the central component. In eukaryotes, the pathway's main function is the degradation of proteins, particularly short-lived proteins that often have regulatory functions, which likely makes the pathway crucial to phenotypes that reflect the diverse niches that *R. oryzae* is found in. Similar to the other two complexes, the ubiquitin–proteasome pathway contains a major complex (proteasome), which together with related genes, constitutes a large number of interacting proteins such that random duplications of individual components could lead to an imbalance of function and ultimately a deleterious outcome. Therefore, Ma et al. argue that in *R. oryzae*, the fact that roughly 80 % of the individual members of the three complexes were retained as duplicates implies whole-genome duplication as the best explanation for the multiple copies of each gene (Ma et al. 2009).

11.6.3 Expanded Gene Families

In addition to the duplication of the large protein complexes described above, a number of gene families appear to have also been expanded in the *R. oryzae* genome, with the affected phenotypes again related to cell growth (Ma et al. 2009). These families include the P-loop GTPases, which regulate basic cellular processes and include homologs of RAS, RHO, CDC, ARF, YPT, as well as other genes that often function as molecular switches that can rapidly coordinate gene expression with varied environmental conditions. A second expanded family is the proteases, which contain the aspartic proteases, subtilisins, caspases, and aminopeptidases among others. These enzymes enhance degradation of organic matter and perhaps most importantly, contain members that are proven virulence factors in other fungi [secreted aspartic proteases, subtilisins (Magee et al. 1993; Monod et al. 2002)]. A third expanded family consists of cell wall biosynthetic genes, in particular the chitin synthases and chitin deacetylases, which is consistent with the high percentage of chitin and chitosan in the cell wall of *R. oryzae* (Ma et al. 2009). The ability to grow rapidly in response to a changing or rich environment requires the fungus to be able to rapidly synthesize new hyphae, which requires substantial amounts of chitin and chitosan, which are encoded by the chitin biosynthetic genes. Finally, in conjunction with cell wall growth, hyphae also need to synthesize the plasma membrane at a rate that keeps up with new cell wall synthesis. Sterols are a major component of cell membranes, and in fungi, the major sterol is ergosterol. The *R. oryzae* genome sequence shows that this fungus, like virtually all fungi, contains all of the components of the highly conserved ergosterol pathway (Ma et al. 2009). Importantly, some of the genes in the pathway, including the major azole target (lanosterol 14α-demethylase), are present in multiple copies, which may affect response to the azole antifungals.

11.7 Genetics and Molecular Biology

Because of the commercial and medical importance of *R. oryzae*, there has been, and continues to be, an interest in the development of the molecular biology and genetics of this fungus. Unfortunately, for the major fungal pathogens that have developed molecular and genetic tools, *R. oryzae* arguably possesses the most problematic aspects of each of the major fungi, with the possible exception of it being dangerous to work with. Since *R. oryzae* is a filamentous mold, a spore preparation must be made, which is more cumbersome than growing yeast cells. Additionally, since numerous genes of the genome have been duplicated, depending on the target, multiple disruptions may be needed, which is cumbersome since there are few markers for this fungus. The transformation systems utilize either biolistics, which requires expensive instrumentation, or spheroplasting, which requires protoplasting enzymes that can be hard to obtain, and a laborious

protocol. There are few genetic markers and the fate of transforming DNA, depending on the input molecule, often is a large multimeric plasmid or concatenated molecules that may or may not be rearranged, both of which have a high tendency to remain episomal. Homologous integration is possible, but of low frequency. However, in spite of numerous inefficiencies of the "molecular toolbox," most of the common molecular and genetic manipulations used in a laboratory are possible with *R. oryzae*, but need to be improved. Importantly, virtually all of the major human fungal pathogens (i.e., *Candida albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Aspergillus fumigatus*, *Coccidioides immitis*) presented large challenges that needed to be overcome before a working molecular system could be developed. But for each of these fungi, once a critical mass of researchers was established, the major hurdles were rapidly overcome.

11.7.1 Transformation

Transformation of *R. oryzae* is possible and has been reported to be successful using the biolistic method, $CaCl_2$/PEG, and *Agrobacterium tumefaciens* (Skory 2002; Michielse et al. 2004). Skory used a uracil auxotroph that was a *pyrG* (orotidine monophosphate (OMP) decarboxylase) mutant as a host for transforming DNA, which consisted of the wild-type *pyrG* gene released from a plasmid by restriction enzyme digestion (Skory 2002). The transformation frequency was low, from 1 to 50 transformants per μg of DNA; however, this number is high enough to perform some basic molecular manipulations and can probably be increased by multiple transformation events. The transformation host in Skory's study was germinated spores, as is typical for filamentous fungi. However, a clear problem with this system is the lack of general integration and as a result, a lack of homologous integration. This problem is not unheard of in fungal pathogens. Both *Cryptococcus neoformans* and *Histoplasma capsulatum* have similar problems since transforming DNA tends to remain episomal but is able to replicate without a clearly defined ARS-like or 2 μm-like origin of replication. For both fungi, incoming DNA is telomerized; however, it is unknown whether or not this is the fate of transforming DNA in *R. oryzae*.

Transformation with circular plasmids was found to rarely lead to integration; however, homologous integration can occur, which is Type I in nature (Hinnen et al. 1978). Interestingly, for non-integrated DNA, circular plasmids are maintained intact and can be recovered in *E. coli* without any apparent rearrangements, suggesting that the original native plasmid was replicated as a circle, although these molecules may be multimeric. Linearized plasmid DNA usually resulted in large multimers of concatenated molecules, which had been repaired by the host cell. When the *R. oryzae pyrG* gene was released from the vector with restriction enzymes that only cut within the vector, homologous integration was the primary outcome, suggesting that the transformation system can be improved. For example, in *C. neoformans*, which tends to maintain transforming

molecules episomally after telomerization, integration can be forced to occur at a higher frequency by using variations of transforming constructs such as a PCR-generated split marker (Fu et al. 2006).

In addition to biolistic transformation, Michielse et al. were able to successfully employ $CaCl_2$/PEG-mediated transformation of spheroplasted spores and a heterologous uracil marker derived from *Aspergillus nidulans* to recover recombinants (Michielse et al. 2004). Importantly, in this same study, they were able to adapt a dominant marker as a transformation system. The dominant marker was the *Aspergillus nidulans* acetamidase gene, *amdS*, which allows transformants to grow on acetamide, providing selection if the recipient strain does not carry a homolog of the gene (Kelly and Hynes 1985). The outcome of transformation with either marker and with the $CaCl_2$/PEG-mediated transformation approach again was to yield primarily episomal transformants. Although the tendency to remain episomal can result in a reduced frequency of homologous integration, this inefficiency is preferable to a high frequency of ectopic integration. Consequently, although the transformation frequency per μg of DNA is somewhat low, there are avenues for improvement that should be achievable using other fungal transformation strategies.

11.7.2 Mutagenesis

One of the most important steps in developing an efficient molecular toolbox is the ability to create and select for mutants. A number of methods have been described for *R. oryzae*. Skory described the use of the chemical mutagen, NNG (1-methyl-3-nitro-1-nitrosoguanidine), as a way to enhance lactic acid production in *R. oryzae* (Skory et al. 1998), while Purohit et al. used NTG (3-nitro,5-methylguanidine) mutagenesis to recover a mutant enhanced for tannase production (Purohit et al. 2006). Bai et al. used diethyl sulfate (DES) to recover mutants that overproduced lactic acid (Bai et al. 2004). Chemical mutagenesis is among the oldest methods for creating useful mutants in virtually any microbe studied in the laboratory. However, there are two concerns when using chemical mutagens. First, the mutagenic nature of the chemical itself can make it hazardous to work with. Second, chemical mutagenesis is random and can lead to a second unlinked mutation elsewhere in the genome that may be undetected, but still exert a phenotype. Having a working genetic system that allows for backcrossing to a wild-type strain in order to reduce the likelihood of the strain of interest carrying a second mutation is helpful. This method would be possible with *R. oryzae* since sexual reproduction has been known to occur; unfortunately, good tester strains that are easy to work with and yield progeny spores at high frequency are not yet available.

An alternate method that is less hazardous but still is a random mutagenic method utilizes UV radiation to create mutants. UV light can be employed in safely enclosed containers to avoid laboratory exposure. Once a kill curve is established, mutagenesis then can be performed on however many colony-forming units are

plated. UV mutagenesis has been used to recover mutants enhanced for tannase (Purohit et al. 2006), lactic acid (Bai et al. 2004), glucoamylase (Suntornsuk and Hang 2008), and fumarate production (Huang et al. 2010). Importantly, for UV mutagenesis and, in general, for any random mutagenesis, it is important to have a good screening technique that can be used to discriminate a desired mutant from a background of nonmutants and non-desired mutants with high efficiency.

11.7.3 Markers, Reporters, and Inducible Promoters

Once a transformation system has been developed for any model organism, useful molecular reagents must follow that expand what is possible to study at the molecular level. These reagents typically include vectors that have regulatable promoters, additional markers that confer transformation flexibility, including the ability to complement disrupted genes, and reporters that allow investigators to measure or locate gene expression or protein function. The molecular toolbox of *R. oryzae* has most of these characteristics, with new reagents continually expanding this versatility.

In many fungi, an early tool is development of markers that are useful for transformation or genetics but also have counterselection capabilities. Arguably the most common of these markers are two mutations in the uracil biosynthetic pathway: orotate phosphoribosyltransferase and orotidine 5′-phosphate decarboxylase (*Saccharomyces cerevisiae URA3* and *URA5* genes, respectively). The value of these genes as markers lies in their ability to be selected for by growing on media containing the toxic analog 5-fluoroorotic acid (5-FOA). Wild-type enzymes of orotate phosphoribosyltransferase and orotidine 5′-phosphate decarboxylase incorporate 5-FOA into pathway intermediates and are killed (Boeke et al. 1984). Both the orotate phosphoribosyltransferase (*pyrF*) and orotidine 5′-phosphate decarboxylase (*pyrG*) have been cloned in *R. oryzae* (Skory and Ibrahim 2007; Skory 2002). Importantly, because of the counterselectability of 5-FOA against functional *pyrF* and *pyrG* genes, it is possible to recycle either mutation in a host strain by selection for loss of the transforming selectable marker if it is *pyrF* or *pyrG* because cells that have the marker are killed by the compound. In the case of *R. oryzae*, which has a tendency to maintain transforming DNA as episomally replicating molecules at high frequency, loss of the transforming construct and marker can be selected for very fast as the construct can be easily lost from the cell because it is not integrated. Similar to this system is the *amdS* system, which uses the compound acetamide, the gene *amdS*, and the toxic analog fluoroacetamide to function as a selection–counterselection system (Debets et al. 1990). The *amdS* gene has been shown to work as a selectable marker in *R. oryzae*, although counterselection with fluoroacetamide has not been reported (Michielse et al. 2004). Another advantage of the *amdS* gene is that it is a heterologous dominant marker, with no *R. oryzae* homolog, which can function to reduce integration in the genome due to the absence of homology.

A third marker that has been reported for *R. oryzae* is a homolog of the *S. cerevisiae his3*, although it has not been characterized (Ibrahim et al. 2010).

Following the development of a transformation system, secondary tools usually follow and enable a deeper investigation of gene function. These secondary tools typically consist of reporter genes and vectors that have regulatable or inducible promoters (Mertens et al. 2006). In *R. oryzae*, multiple promoters have been described, and one reporter gene has been shown to function by Mertens et al. (2006). In this study, they used a vector containing the *R. oryzae* pyruvate decarboxylase (*pdcA*) promoter and terminator, the *R. oryzae pyrG* gene as selectable marker, and pBluescript II KS- as the plasmid backbone. Two additional promoters were tested, *pgk1*, from the phosphoglycerate kinase 1 gene, and *amyA*, from the glucoamylase A gene, by swapping them into the vector in place of the *pdcA* promoter. The three promoters were selected to test different types of regulation using GFP as a reporter; the *pgk1* promoter is constitutive, *pdcA* promoter is inducible by glucose, and *amyA* is highly inducible by starch or cellobiose. All three promoters were found to be functional as evidenced by observation of GFP fluorescence, with expression levels being the highest in *pdcA* and lowest in *pgk1*.

11.7.4 Agrobacterium tumefaciens-*Mediated Transformation*

Agrobacterium transformation is becoming an increasingly powerful tool in mycology, with virtually every fungus in which it has been tried being transformable (McClelland and Wickes 2009). The major significance of this bacterium in fungal molecular biology is that it is a transkingdom pathogen that can move DNA into cells with very little specificity. The system works by simply taking the *A. tumefaciens* native Ti plasmid, inserting a selectable marker, and then transforming the plasmid back into the *A. tumefaciens* host. The plasmid still maintains its infectivity and can be transferred into the target host by appropriate co-culture conditions in which the bacterium is mixed with the fungal host under conditions that induce plasmid mobilization. After allowing time for the plasmid DNA to be inserted into the host fungal cells, the bacterial–fungal mixture is harvested and then replated under conditions that include an antibiotic to kill residual *A. tumefaciens* and an antibiotic (in the case of the marker being drug resistance) or minimal medium (in case the marker complements an auxotrophy) to select for transformed fungi. The outcome of this infection is predominantly integrated transformants, usually in single copy, which makes this method of transformation extremely powerful for fungi that have inefficient or difficult transformation systems.

In an effort to overcome the tendency of transforming DNA in *R. oryzae* to remain episomal, Michielse et al. (2004) developed an *Agrobacterium*-mediated transformation system for *R. oryzae*. Their reason for exploring

Agrobacterium-mediated transformation centered around developing a transformation method that resulted in stable transformants, which typically occurs after integration of transforming DNA. They used a $CaCl_2$/PEG method for comparison of transformation fates. They found, using the *Agrobacterium*-mediated method, that integration of transforming DNA occurred 100 % of the time whereas the $CaCl_2$/PEG method using linear or circular molecules resulted in a <5 % integration frequency regardless of selectable marker. Further investigation concluded that the transforming DNA integrated at a hotspot; however, they were unable to identify the locus. Importantly, experiments were not performed to see if this method could be used for gene disruption, which would be the real value of an *Agrobacterium*-mediated method since it appears that this method solves the problem of episomal maintenance of transforming DNA.

11.7.5 Gene Disruption

Much of the investigation into transformation systems is needed in order to obtain gene disruptions at high efficiency, or at least at a level where a disruptant can be detected after a simple PCR screen of multiple colonies. Screening 10 or 20 colonies after a transformation is not hard, but as this number gets to be 50, 100, or higher, obtaining a disruptant becomes laborious, and it becomes further complicated because one can never be sure they are not dealing with an essential gene that cannot be disrupted.

A major issue that affects disruption frequency is the fate of transforming DNA. If it is difficult to get DNA to integrate, this problem is the most important hurdle to overcome. Presently, integration frequency is a challenge for *R. oryzae* transformation. A second major issue is homologous integration frequency. If integration frequency at the target locus is very low, screening for disruptants again becomes laborious, which becomes grossly inefficient if integration frequency is low to begin with. These are current challenges in *R. oryzae* molecular biology; however, there have been some successes. Ibrahim et al. were able to disrupt the *R. oryzae FTR1* gene by using a simple transformation construct in which the marker gene, *pyrF*, was flanked with *FTR1* sequences that lie outside the *FTR1* coding region, thereby targeting the disruption cassette to the *FTR1* locus due to flanking sequence homology (Ibrahim et al. 2010). This technique worked, as a clean replacement of the *FTR1* coding sequence occurred. However, another problem with *R. oryzae* transformation was revealed. Almost all zygomycetes are coenocytic, which means that there are no septa in the hyphae. Consequently, all of the transformation host cells are multinucleate. Since the transforming DNA is only going to enter a single nucleus, and the probability is likely low that all of the nuclei of the same cell will be transformed, transformed cells must undergo posttransformation manipulation, usually in the form of subculturing, in order to recover a homokaryon. In the case of *FTR1*, creating a homokaryon was not successful.

Given the ever-present problem of dealing with heterokaryons and essential genes, another method to study gene function is needed. Based on results from the genome sequence, it was predicted that *R. oryzae* contains the genes that would allow RNAi to work as a gene disruption method. The genome is predicted to contain two copies of Argonaut, one Dicer homolog, and five RNA-dependent polymerases (RdRP) (Ma et al. 2009; Meussen et al. 2012). This information was used to develop an RNAi construct, which was successfully used to knockdown *FTR1* expression (Ibrahim et al. 2010), therefore adding an important tool to the molecular biology of *R. oryzae*.

11.8 Conclusions

R. oryzae is currently the model fungus for the study of the pathogenic zygomycetes. The present genetic and molecular system is clearly in its infancy and currently seems laborious and inefficient. However, the molecular biology of all the major human fungal pathogens started off in the same way, and some still have residual problems that have remained after more than 20 years of investigation. However, knowledge from these systems can be applied to new model fungi to help establish working models. Continued investigation then ultimately leads to improvements until virtually all required manipulations become possible. In the case of *R. oryzae*, this fungus is becoming increasingly important as a pathogen and remains an important commercial and food industry organism. All of these areas require continued advancement of what we know about this fungus, which will attract more investigators into the field who will collectively be able to solve current and future problems associated with the study of *R. oryzae*.

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Chapter 12
Podospora anserina: From Laboratory to Biotechnology

Philippe Silar

12.1 Introduction

In a typical grassland or savannah, a large part of the plant biomass ends up in the digestive tracks of animals such as herbivorous mammals or birds. However, these animals are able to retrieve only part of the nutrients present in their food and release in the form of dung, a ready-to-use meal already invaded when deposited on the ground by many fungi, the coprophilous fungi. These are usually present in form of spores in the plant diet, germinate while passing through the digestive track and, after growth, have evolved special designs to disperse their spores, so as to maximise the chances that they are ingested by another herbivore. Apart from the chytrids, species from all major fungal taxa may grow and fruit on dung. Usually, they appear in a succession that somewhat reflects the evolution of terrestrial Eumycota fungi, starting with Mucoromycotina (e.g. Mucor, Phycomyces and Pilobolus) followed by basal Pezizomycotina such as Orbiliomycetes and Pezizomycetes, then supposedly more advanced ones such as Dothideomycetes, Eurotiomycetes, Leotiomycetes and Sordariomycetes. The sequence finishes with some Basidiomycota belonging to the Agaricomycetes. The succession likely reflects a combination of choices in life strategies and enzymatic equipments selected during evolution, whereby the first to appear have very little competitive ability and are able to use moderately complex carbohydrates polymers but grow and fruit fast, while the later ones use more complex food sources, i.e. lignocellulose, and have antagonistic abilities enabling them to grow and fruit slower in dung. Overall, these fungi are part of a food chain that enables materials

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_12,
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contained in droppings to go back to the soils, rendering them fertile. Indeed, what better fertiliser than manure?

Among the 2–3,000 fungal species that inhabit dung obligatorily or facultatively, a few of them have been used as model organisms to study various phenomena ranging from isolation of fungal compounds and meiotic recombination to ageing and cell differentiation. However, three species have mostly been used for these studies: *Podospora anserina*, *Sordaria macrospora* and *Ascobolus immersus*. Table 12.1 presents some of their characteristics in comparison to those of the two most popular fungal models *Aspergillus nidulans* and *Neurospora crassa*. The most salient feature, especially for *P. anserina* and *S. macrospora*, is the shortness of their generation time: it is 2–3 times shorter than that of *A. nidulans* and *N. crassa*, making them excellent tools to rapidly obtain mutants. Yet, they lack conidia, which may impair some studies. However, the development of machines, such as "FastPrep", or efficient protoplast formation enabling fragmentation of mycelia into uninucleated cells makes the lack of conidia a less important drawback than before.

The three coprophilous species have now their whole genome sequence publicly available. *A. immersus* is part of the "1000 fungal genomes" project from JGI (http://1000.fungalgenomes.org/home/). *S. macrospora* has its genome published (Nowrousian et al. 2010) and is mostly utilised to study meiosis (Espagne et al. 2011; Storlazzi et al. 2010) and fruiting body development (Engh et al. 2010). *P. anserina* (Fig. 12.1) is used in a wider array of studies dealing with ageing, sexual development, prions and other non-conventional hereditary units, signal transduction and differentiation, plant biomass degradation, cell fusion and vegetative incompatibility, interactions with other fungi, mitochondrial physiology, translation, secondary metabolism, etc. Moreover, the availability of its genome sequence has enabled the development of several tools, as well as its utilisation in some biotechnology applications. The purpose of this chapter is to review the biology of *P. anserina* and to what extent the availability of genomic and postgenomic tools has furthered the research with this organism.

12.2 The Pre-genome Era

12.2.1 Early History of the Research on P. anserina

Because of the ease of its manipulation, *P. anserina* was chosen very early on as a model, first to study ascospore formation (Wolf 1912) and hyphal fusion (Buller 1933). Later, Georges Rizet worked out the complex genetic analysis resulting from the atypical ascus development linked to pseudohomothallism (Fig. 12.2; Rizet 1939, 1941) and initiated a French school of geneticists that worked with filamentous fungi (mostly *P. anserina* and *A. immersus*). The visit of Karl Esser in Rizet's laboratory (Esser 1954; Rizet and Esser 1953) further fuelled research in Germany, especially with the addition of biochemical studies (Esser 1963). This opened an era

Table 12.1 Main features of popular and not so popular fungal saprobes

	Taxonomy	Lifestyle	Growth speed	Senescence	Asexual spores	Mating system	Generation time	Fruit body type
Podospora anserina	Sordariales Lasiosphaeriaceae	Coprophilous (=fimicolous)	7 mm/day	Early onset	None	Pseudohomothallic	7 days	Perithecium
Sordaria macrospora	Sordariales Sordariaceae	Coprophilous (=fimicolous)	3 cm/day	Late onset	None	Homothallic	7 days	Perithecium
Ascobolus immersus	Pezizales Ascobolaceae	Coprophilous (=fimicolous)	3 cm/day	Unknown	None	Heterothallic	10–15 days	Apothecium
Neurospora crassa	Sordariales Sordariaceae	Carbonicolous (pyrophilous)	7 cm/day	Only in few strains	Micro- and macroconidia	Heterothallic	2–3 weeks	Perithecium
Aspergillus nidulans	Eurotiales Trichocomaceae	Soil and litter saprobe	8 mm/day	Unknown	Conidia	Homothallic	2–3 weeks	Cleistothecium

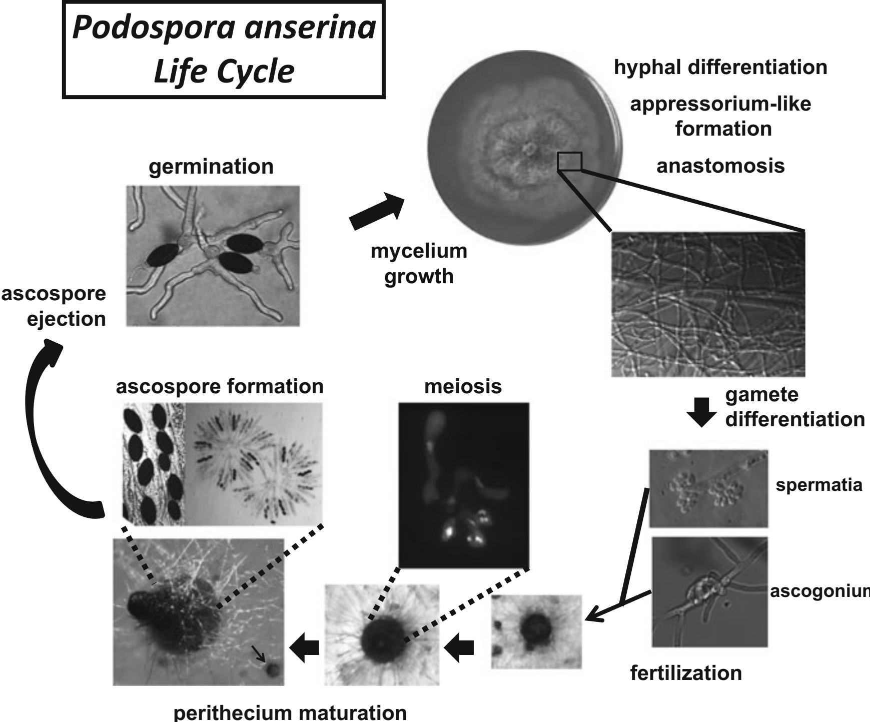

Fig. 12.1 *Podospora anserina* life cycle. See text for details

when several important biological phenomena were discovered and their genetic determinants elucidated.

The "barrage" phenomenon lead to the study of vegetative (heterokaryon) incompatibility and to the discovery of an unusual form of inheritance (Beisson-Schecroun 1962; Rizet 1952; Rizet and Delannoy 1950), which is now known to be due to a prion (Coustou et al. 1997; Maddelein et al. 2002; Ritter et al. 2005; see Sect. 4.2). The discovery that *P. anserina* cannot be cultivated infinitely permitted studies on senescence (Marcou 1961; Rizet 1953) and mitochondrial physiology (Cummings et al. 1978; Jamet-Vierny et al. 1980; Tudzynski and Esser 1977). Note that Rizet published his study 12 years before the seminal paper of Leonard Hayflick disproving immortality of human cells (Hayflick 1965). Among the other topics studied are the first fungal meiotic drive (now known as spore killer; Padieu and Bernet 1967), translation accuracy (Coppin-Raynal et al. 1988; Picard 1973), rhythmic growth (Chevaugeon and van Nguyen 1969; Esser 1969) and fruiting body formation (Esser and Graw 1980; Labarere and Bernet 1979; Lysek 1976). In parallel, field studies of coprophilous fungi showed that *P. anserina* was frequently recovered from dung (Furuya and Udagawa 1972; Lundqvist 1972). At the end of these early studies, the ecology and the life cycle were fully known, sexual reproduction was mastered in the lab on fully defined media, genetic analysis

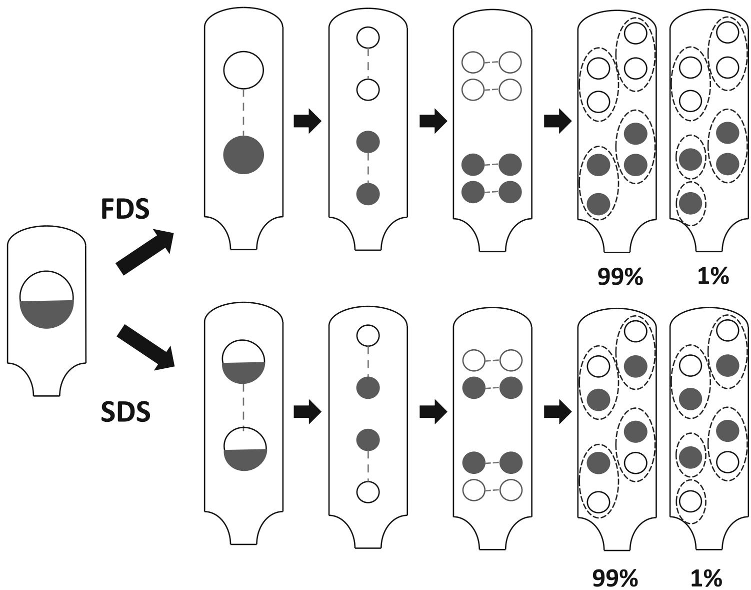

Fig. 12.2 *Podospora anserina* ascus structure. Most asci contain four binucleated ascospores. In a few (~1 %) asci, a "binucleated" (or big) spore is replaced by two "uninucleated" (or small) ones. In Fig. 12.1, enlargement shows a typical 4-spored ascus along with a 5-spored one. FDS asci originate from meiosis in which no crossing over occurs between the marker and its centromere. Each half of the ascus is genetically homogeneous. In SDS asci, one crossing over between the marker and its centromere yields asci having four ascospores heterokaryotic for the marker. FDS to SDS ratio depends upon the position of the marker with respect to its centromere. Centromere-linked marker have a 100 % FDS, while telomere linked may reach almost 100 % SDS (instead of 66 % due to multiple crossing overs), thanks to a crossing-over interference which limit crossing to one in some regions

used routinely (Marcou et al. 1982) and a genetic transformation system was available (Begueret et al. 1984; Brygoo and Debuchy 1985).

12.2.2 Ecology and Life Cycle of P. anserina

Podospora anserina (Ces.) Niessl, also known as *Pleurage anserina* (Ces.) Kuntze or *Podospora pauciseta* (Ces.) Traverso, appears to grow exclusively on dung, although there is one report indicating that it may also live as a plant endophyte (Matasyoh et al. 2011). However, the strain isolated by Matasyoh et al. is not available for crossing, rendering final attribution to the *P. anserina* species difficult. *P. anserina* has been found in many regions, including northern and southern Europe, Brazil, New Zealand, Kenya, etc., on droppings from a large array of herbivore species, suggesting that this species is now cosmopolitan. This lifestyle has two important consequences on *P. anserina* life cycle. Firstly, its reproduction

is purely sexual, which may result from the necessity of actively dispersing its spores away from its dung substrate (herbivores tend not to eat their faeces), a feature easily achieved by perithecia, but not so much by conidiophores. Secondly, its restricted biotope is likely responsible for development of senescence, a feature shared by other coprophilous fungi (Geydan et al. 2012), as in nature *P. anserina* likely never grows enough to actually present senescence. Finally, *P. anserina* is one of the fungi that fruits late in the succession on dung. This is correlated with an enzymatic repertoire that enables it to degrade lignin to access cellulose (Espagne et al. 2008) and a defence mechanism, called Hyphal Interference, which likely permits *P. anserina* to eliminate some competing fungi (Silar 2012).

P. anserina is very easy to grow, and the complete cycle (Fig. 12.1) can be mastered by using two media. The standard growth and crossing medium (M2 or SU) contains dextrin as the carbon source and urea as the nitrogen source. The pH is maintained at 7 by a phosphate buffer and minerals as well as biotin and thiamine are added as traces. On this medium the fungus grows at a rate of 7 mm/day and starts to differentiate male (spermatia) and female (ascogonia) gametes after 3 days. Spermatia are not conidia and they are unable to germinate, although previous reports suggest they may do so in some conditions (Beisson-Schecroun 1962; Esser and Prillinger 1972). Alternatively, hyphal fragment can act as male gamete. If gametes compatible for mating are present, fertilisation ensues and the fruiting body matures in 4 days, yielding a progeny of hundreds of ascospores that are continuously expelled for up to 5 more days. *P. anserina* is pseudohomothallic, meaning that it is formally a heterothallic fungus with two mating types (*mat*+ and *mat*−) and fertilisation is possible only between *mat*+ and *mat*− gametes; however, ascospores are binucleated and nuclei usually contain alternative versions of the mating type. Hence, the mycelium germinating from *P. anserina* ascospores is normally heterokaryotic and self-fertile. This facilitates genetic analysis, because *mat*+ and *mat*− wild-type strains differ only by a small region around the mating-type locus. On standard medium, ascospore germination occurs with a very low efficiency (about 0.01 % if the ascospores are not collected and about 1 % if they are collected on a needle). This is likely due to the fact that in nature, ascospores are triggered to germinate by passage through the digestive tract of an herbivore. This can easily be recreated in the lab, by incubating the ascospores on a medium containing ammonium acetate and Bacto Peptone. Ascospores transferred one by one at the tip of a needle on this medium germinate at nearly 100 % efficiency. All protocols to work with *P. anserina* are currently available at http://podospora.igmors.u-psud.fr/. This site also hosts the latest version of the *P. anserina* genome as well as information and bibliography on this organism.

12.2.3 Doing Genetic Analysis with **P. anserina**

The early studies identified many mutants, obtained spontaneously or induced by UV or chemical treatments, and segregation analyses resulted in a genetic map with

nearly 150 markers located on seven nuclear linkage groups (Marcou et al. 1982) and a mitochondrial chromosome (Belcour et al. 1978), which was in line with the seven chromosomes observed during meiosis (Beckett and Wilson 1968; Franke 1962).

Although in decline during the 1980s and 1990s, identification of mutants affected in various processes is still going on (Haedens et al. 2005; P. Silar, unpublished data) and, thanks to the new "next-generation sequencing" (NGS) technologies, may regain favour. To this end, UV mutagenesis procedures are now improved, owing to easier protoplast recovery and new tools for mycelium fragmentation. Indeed, protoplasting (Belcour 1976) results in formation of ~5 μm roundish cells, many of which appear uninucleated. Typically, 10^4 protoplasts are spread delicately onto a Petri plate containing M2 medium osmostabilised with 200 g/L of sucrose, irradiated with by 200–300 J/m^2 of UV at 254 nm (such as those produced in cross-linkers for DNA) and left in the dark overnight. Typically, around 100 thalli regenerate after 1 or 2 days from such plates and can be replica plated individually to screen mutants as desired. Alternatively, for direct screens, such as those of suppressors of mutants affected in sexual reproduction, mycelium can be fragmented in a "FastPrep" or similar machine, spread onto M2 plates and regenerated overnight. This yields plates finely covered with a mycelium veil that can then be irradiated as above. Note that additional methods of mutagenesis may be used depending on the mutants desired.

The next step of mutant analysis is mapping, which thanks to *P. anserina* ascus structure (Fig. 12.2) is quite simple: ordered tetrad analysis can be made from unordered asci! A cross with wild type enables to check if the mutant phenotype is caused by a single mutation by observing 2:2 segregation on small ascospores or five-spored asci. First-division segregation (FDS) and second-division segregation (SDS) analysis from the same cross permits a first rough mapping of the mutation with respect to the centromere, and analysis of the phenotypes of strains issued from SDS asci gives some clue as to whether the mutation is dominant or recessive. Moreover, heterokaryon formation is very straightforward and rapid in this species: mixing of mycelium fragments results in a heterokaryotic culture overnight, which facilitates dominance/recessivity and "complementation" tests. To locate mutations more precisely, the genetic map of *P. anserina* (Fig. 12.3) is now replete with markers, often generated by gene replacement with a dominant resistance marker to antifungal substances (see next section). Alternatively, we now have the complete genome sequence of the distantly related "T" strain available (P. Silar et al. unpublished data). This strain presents numerous polymorphisms with the S ("big S") and s ("small s") strains that are typically used to screen for mutants, the S strain being the first one sequenced (Espagne et al. 2008). Analysis of polymorphic markers in a S $\times$ T cross has confirmed and refined the assembly of the genome of the S strain (Espagne et al. 2008), and similar analysis has been used to identify mutations through positional cloning (Adam et al. 2012; Espagne et al. 2011; Sellem et al. 2009). New-generation sequencing techniques now allow for more rapid identification of the mutant genes (see Sect. 4.1).

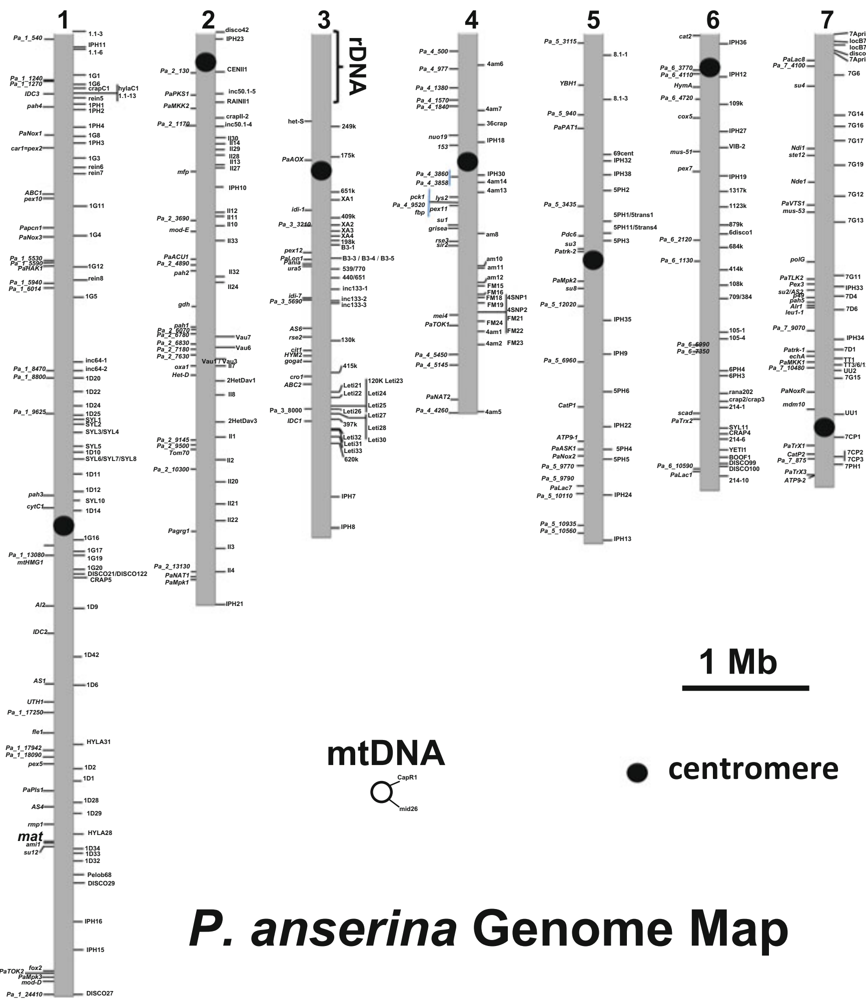

Fig. 12.3 *Podospora anserina* genome map. On this map of strain S genome, which is in scale with the sequence and not the genetic distances, classical genetic markers are on the *left* and polymorphisms (mostly microsatellites and indels) with strain T on the *right*

Mutants obtained through genetic screens are stored easily at −80 °C, simply by adding small 2 mm × 2 mm × 2 mm mycelium explants to 1 ml of RG medium, which is M2 medium supplemented with 200 g/L of sucrose. In such conditions, wild type and mutants can be frozen and thawed several times without loss of vigour. This method may be of widespread utilisation as in our hands all Eumycota (alas not Oomycetes) can be safely stored for an extended period of time (our oldest strains have been stored for now nearly 20 years).

12.2.4 Genetic Transformation of P. anserina

Early studies also yielded an efficient transformation system. It is based on the transformation of protoplasts with the help of PEG. It does not require complex centrifugation steps in sucrose or KCl gradients for protoplast purification, and those can been stored for an extended period of time before utilisation. The first description of transformation was made with a plasmid, which lacked a dominant marker (Stahl et al. 1982). Then, systems using either uracil auxotrophy (Begueret et al. 1984) or suppressor tRNAs correcting leucine auxotrophy (Brygoo and Debuchy 1985) were developed. Now, dominant makers conferring resistance to drugs are usually used. Four are routinely used in our lab, resistance to hygromycin B, phleomycin, nourseothricin and geneticin, and special vectors have been designed for easy cloning, firstly with hygromycin B and phleomycin (Silar 1995) and now with nourseothricin and geneticin (H. Lalucque and P. Silar, unpublished data). Further improvements on this transformation system have been made to delete or replace genes since the availability of the genome sequence [(El-Khoury et al. 2008) and see Sect. 3.3].

12.3 The Genome of *P. anserina*

12.3.1 The Roadmap Towards the Genome Sequence

With the release of the *Saccharomyces cerevisiae* genome sequence and the proposals for sequencing other genetic models (*Escherichia coli*, Drosophila, Arabidopsis, etc.), the French Podospora community decided very early on (in 1999) to sequence the *P. anserina* genome in order to access new tools that would facilitate research with this model. A sequence of the complete mitochondrial genome was already available since 1990 (Cummings et al. 1990), and for a long time it was the longest contig present in the GenBank database! The first pilot project aimed at sequencing the region surrounding the centromere of chromosome 5 was proposed in 1999 and accepted in September 2000 by Genoscope, the French sequencing agency. Five bacterial artificial chromosomes covering 500 kb around the centromere were completely sequenced and manually annotated (Silar et al. 2003). These first results showed that the *P. anserina* was likely to be poor in repeated sequences and introns and defined consensus for intron splicing and translation start sites.

The pilot study was then followed with acceptance by Genoscope in 2002 of a 0.5-fold coverage of the genome (at that time the *N. crassa* genome became available and could be used to help with assembly) and in 2003 of the complete sequence with a 10-fold coverage. The project was also proposed by the Broad Institute (at that time the Whitehead Institute) in its “October 10, 2003, White Paper” of the Fungal Genome Initiative, and after agreement between the two

agencies, Genoscope proceeded with sequencing and assembly. 33 large scaffolds comprising 1,196 contigs were obtained along with a few more small ones devoid of unique regions. To help annotation, about 50,000 cDNA were also sequenced from both ends. Annotation was custom-made with a mixture of comparative predictions, using the *N. crassa*, Swiss-Prot and GenBank data, mapping to the sequenced ESTs and de novo prediction, as, at that time, purely de novo prediction programmes did not perform very well. The genome sequence and its analysis were published in 2008 (Espagne et al. 2008). Since then Genoscope has generated sequences with the 454 technology and over 20-fold coverage, and we have generated over 100× coverage of the genome with the Illumina technology. This has enabled us to correct numerous sequencing errors (P. Silar et al. unpublished data). A few remaining gaps located in unique sequences were manually filled by PCR amplification and sequencing of the recovered products. Today, the genome is thus assembled into seven large scaffolds corresponding to the seven nuclear chromosomes and a small one corresponding to the mitochondrial genome (Fig. 12.3). All scaffolds are free of gaps in the unique regions (but unfortunately not in repeated ones!), and resequencing shows that they contain minimal amounts of sequence errors (some regions rich in similar bases may still contain a few errors). Manual examination of the annotation has also enabled us to correct mistakes, to reclassify some predicted CDS as pseudogenes and also to create new CDS. Although not yet perfect, the community has now at its disposal a very good quality annotated sequence. All data are available at http://podospora.igmors.u-psud.fr and are updated frequently.

12.3.2 Main Features of the Genome Sequence

The *P. anserina* genome has a size of about 35.5 Mb, which is similar to that of related species, and a GC content of about 50 %. Presently, it contains 10,635 CDS, most of which are only “predicted” and not fully ascertained by any other means afforded by the gene-by-gene analysis. However, microarray analysis evidenced expression for most of them (see Sect. 3.3). Such number is typical for filamentous fungi. Comparisons show that *N. crassa* and *P. anserina* share about 6,800 genes with an average of 60 % identities in orthologous CDS. Each species has thus nearly 3–4,000 CDS, which are not present in the other species (Espagne et al. 2008). Actually, *P. anserina* and *N. crassa* genetic divergence is more pronounced than that of mammals and fishes! This is also reflected by the overall lack of gene synteny between the two species, most genes being on the same chromosomes but in different positions and orders: the largest synteny block is 37 genes surrounding the mating type, in a region devoid of recombination during meiosis in *P. anserina* and *N. crassa* (Espagne et al. 2008). Despite the widespread belief that *P. anserina* is a facsimile of *N. crassa*, the genome data clearly show that it is not the case, which reflects their completely different lifestyles (Table 12.1). Like the genes of the other Pezizomycotina, *P. anserina* genes are tightly packed with often less than

1 kb between consecutive genes. They contain few introns (frequently none or a single one) of short sizes (~50–60 nucleotides), and some alternative splicing, antisense RNA and "non-coding" transcription units have been evidenced (Espagne et al. 2008).

The total amount of the repeated regions is unknown because telomeres, centromeres and the rDNA are poorly assembled and a few short scaffolds comprising only repeated regions are not yet incorporated into the assembly. Several class I and class II transposons are present, often in clusters associated with segmental duplication, and represent about 3.5 % of the genome. Lack of obvious mutations in the CDS of the transposases and the reverse transcriptases in some copies suggests that some are still functional. Confirming this, the de novo insertion of a "rainette" DNA transposon has recently been detected (M. Dequard-Chablat, personal communication). Segmental duplications represent about 1.5 % of the genome and are nearly always close to transposons, suggesting that they are likely associated with incorrect transposition events. Most repeated sequences have traces of repeat-induced point mutations (RIP) showing that in the long term this phenomenon is indeed able to inactivate potentially harmful sequences, although in *P. anserina* this phenomenon is much less efficient than that in *N. crassa* (Bouhouche et al. 2004; Coppin and Silar 2007; Graïa et al. 2001). This lesser efficiency is correlated with the presence in the genome of a few multigenic families, including the Het genes involved in vegetative/heterokaryon incompatibility, whose evolution may even be fuelled by RIP (Paoletti et al. 2007). Finally, a few repeated sequences of unknown affinities have been discovered, some of which could code for RNAs with various functions (Espagne et al. 2008).

Overall, the genome of *P. anserina* does not present any obvious structural features that would make it different from those of the other Pezizomycotina. In this sense, *N. crassa* with its strong RIP may be less typical than *P. anserina* (Galagan et al. 2003). With respect to its coding content, *P. anserina* appears to possess classic amounts of genes for primary and secondary metabolism (see Sect. 4.3), transporters, signalling, cytoskeleton, basic molecular biology processes as well as a large proportion of CDS with unknown functions that await characterisation.

12.3.3 Tools Created by the Genome Project

In addition to providing important information on the biology of *P. anserina*, the genome project has also generated a set of tools accelerating research with this organism. Firstly, an interactive website dedicated to the fungus (http://podospora.igmors.u-psud.fr) was set up. On this site, information on *P. anserina* is available, and BLAST analyses against the genome, the predicted CDS, the EST dataset and the raw sequence traces can be performed. Chromatograms are available at the NCBI Trace Archive if needed. Moreover, the *P. anserina* genome data have been

integrated to the FUNGIpath resource (Grossetete et al. 2010), which enables rapid location of metabolic pathways of *P. anserina* and orthologous gene in other fungi.

All DNA banks generated by the project, including 3-kb, 12 kb, BAC and cosmids are available for research purposes and are used, for example, in transformation experiments. cDNAs of the EST project are also available. Some are cloned into a yeast expression vector under the control of the strong PGK promoter and may be directly transformed into *S. cerevisiae* for transgenic expression (Espagne et al. 2008).

The genome assembly also required verification, which was done by establishing a new genetic map with more than 120 polymorphic markers (Espagne et al. 2008). These markers, along with some older ones (Coppin and Silar 2007; Dequard-Chablat and Silar 2006) and numerous new ones discovered since (Fig. 12.3), are now available for mapping genes during positional cloning (Adam et al. 2012; Espagne et al. 2011; Sellem et al. 2009) and also for locating crossing overs (Espagne et al. 2011).

12.4 After the Genome: New Tools for Fundamental and Applied Research

12.4.1 New Tools Created After the Genome Project

Since the release of the genome sequence, additional tools have been developed. Firstly, strains devoid of their *mus51* gene involved in non-homologous recombination were constructed by replacing the *mus51* coding sequence with a phleomycin resistance marker (El-Khoury et al. 2008) or the *su8-1* suppressor tRNA (Lambou et al. 2008). As described earlier in *N. crassa* (Ninomiya et al. 2004), recombination proceeds almost exclusively between homologous regions in these strains, which facilitates gene inactivation. Without *mus51* inactivation, plasmid transformation could be messy with tandem insertions of several copies often truncated (Razanamparany and Bégueret 1988; Rossignol and Silar 1996). Nevertheless, cosmids containing 40 kb of genomic DNA often integrate through homologous recombination in wild type (Picard et al. 1987). However, these integrations are unstable and excise during meiosis by a single crossing over event (Coppin-Raynal et al. 1989; Picard et al. 1987). For gene deletion, many strategies may be followed, including one similar to the high-throughput method used for inactivating the whole set of *N. crassa* genes (Bidard et al. 2011; Colot et al. 2006). In our lab, the strategy that we found most effective is the "split marker" described in Fig. 12.4. This method requires two successive PCR reaction sets and the co-transformation of two DNA fragments carrying part of the deletion cassette. *P. anserina* finishes the job by making three homologous recombination events. In a typical experiment, 5–50 transformants may be recovered, over 80 % of them being correctly inactivated, as judged by Southern blot analysis. Because the

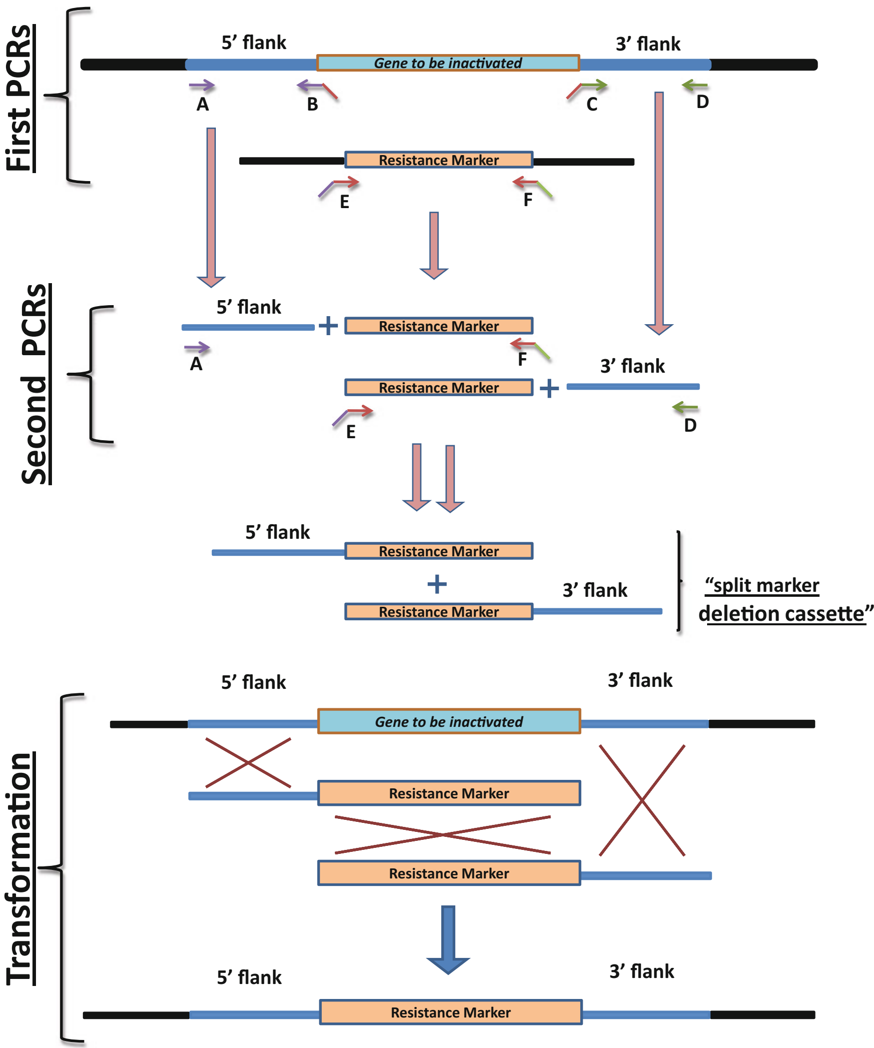

Fig. 12.4 Gene inactivation with the "split marker" method. In the first step, two PCR reactions amplify the 5′ and 3′ flanking regions of the gene to be deleted using primer couples (A, B) and (C, D) respectively; a third PCR reaction amplifies the resistance marker using primers E and F. To enable fusions, primers B and C and primers E and F contain complementary sequences. In the second step, two separate PCR reactions fuse the 5′ flanking fragment with the resistance marker and the other the 3′ flanking fragment with the resistance marker using primer couples (A, F) and (E, D), respectively. A 2-piece deletion cassette is obtained with the resistance marker with either one of the flanking region: the split marker deletion cassette. The two fragments are introduced together by transformation into *P. anserina*. Thanks to three recombination events (*red crosses*), the gene is then deleted and replaced by the resistance marker

sexual cycle is so easy to undergo, the primary transformants are backcrossed to the wild type, and "clean" homokaryotic strains inactivated for the gene and devoid of the *mus51* inactivated allele are recovered in the progeny.

Using the *mus51*-deleted strains, green fluorescent protein (GFP) tags may also be added through a single recombination event at the end of any gene enabling to localise the protein through GFP fluorescence (Malagnac et al. 2013). Interestingly, in this case the tagged protein is expressed thanks to the natural promoter of the gene, which remains in its proper chromosomal location. In addition, two vectors that target the integration of exogenous DNA at two defined loci in single copies have been created to use with *mus51* deletion (Déquard-Chablat et al. 2012). They should permit to better monitor construct expression and make, for example, comparison between alleles more straightforward.

Down to single nucleotide, changes within the *P. anserina* genome may also be introduced using the *mus51*-inactivated strains (El-Khoury et al. 2008). A strain carrying both mating-type loci in tandem has been created this way (E. Coppin and R. Debuchy, personal communication). In this strain, the *mat*+ idiomorph was introduced, without additional sequences, upstream of the mat− idiomorph, recreating a composite mating-type locus resembling that of the homothallic *S. macrospora* (Poggeler et al. 1997). This PM154 strain is self-fertile as a homokaryon and can also fertilise mat+ and mat− strains: however, meiosis and spore formation are not as efficient as in a *mat*+ × *mat*− crosses (likely because haploid nuclei engage in meiosis and abort). It is presently used to select for recessive mutations affecting fruiting body formation (P. Silar, unpublished data).

Agilent microarrays have also been designed and optimised for the 10,556 CDS defined by the genome project (Bidard et al. 2010). These are able to probe with high statistical significance the transcriptional expression of more than 10,000 genes (Bidard et al. 2012). They have been used to detect genes differently transcribed between the *mat*+ and *mat*− strains (Bidard et al. 2011), during mycelium growth (Bidard et al. 2012), perithecium development (V. Berteaux-Lecellier and F. Bidard, personal communication) and ascospore germination (M. Dequard-Chablat, personal communication). We thus now have an overview of the transcriptomes of *P. anserina* during its major developmental stages in laboratory conditions.

With the advances of the new sequencing technology (Next Generation Sequencing or NGS), resequencing genomes is affordable and turns out to be the cheapest way to identify mutations. To this end, UV-induced mutants are backcrossed five times with the wild type to eliminate spurious mutations. Genomic DNA is then sequenced in multiplex (e.g. a single Illumina lane can be used to sequence the genome of three strains with coverage of more than 40-fold, which is more than enough to detect mutations). Moreover, strains carrying two different mutations to be identified may be constructed, which permits to cut costs even more. Analysis with the SAMtools package (Li et al. 2009) efficiently identifies the mutated sequence in the generated sequences. If several candidate mutations are detected, genetic mapping analysis may help to narrow the search for the true culprit (see Sect. 2.3). Final identification can be made using the molecular tools

already available for *P. anserina*; especially, transformation with a DNA fragment encompassing the actual mutation should restore a wild-type phenotype (if the mutation is recessive!). Papers in which mutations are identified this way should be published shortly (P. Silar et al. unpublished data). In comparison, the sib selection (e.g. Berteaux-Lecellier et al. 1995; Turcq et al. 1990) and positional cloning methods (Adam et al. 2012; Espagne et al. 2011; Sellem et al. 2009) used previously are more time consuming and expensive. This should foster the utilisation of forward genetics, instead of reverse genetics, to gain insight into the mechanisms of many biological processes as presented in the next section.

12.4.2 *The Main Areas that Benefit from the Research with* P. anserina

With nearly 600 publications indexed in “PubMed”, including nearly 250 from the last 10 years, research with *P. anserina* is fairly active. It is thus impossible in the scope of this chapter to present a comprehensive review. Only active fields of research with the most relevant references will be briefly presented.

The first major field of research, both historically and in terms of publication, is senescence. The early works (Marcou 1961) suggested that this phenomenon is triggered by the appearance and subsequent exponential amplification of a cytoplasmic and infectious “determinant”, whose nature is at the present time still unknown (Jamet-Vierny et al. 1999). However, cytosolic translation (Belcour et al. 1991; Silar et al. 2001) and especially mitochondria (Cummings et al. 1979; Jamet-Vierny et al. 1980; Tudzynski and Esser 1977) have been implicated as major factors. This has led to numerous studies that connect mitochondrial physiology, including its role in apoptosis, with longevity control and abnormal mitochondrial DNA molecule accumulation (e.g. Adam et al. 2012; El-Khoury and Sainsard-Chanet 2010; Rexroth et al. 2012; Scheckhuber et al. 2011; Sellem et al. 2009; van Diepeningen et al. 2010). Respiration is likely one of the key factors for senescence, as diminution of the respiratory activity leads to increased lifespan (Dufour et al. 2000; Sellem et al. 2007) and restoration of the electron flow by an alternative oxidase restored senescence (Lorin et al. 2001). Most research is relevant to general ageing as the factors discovered in *P. anserina* are likely to be conserved in human (Fischer et al. 2013; Osiewacz et al. 2010; Scheckhuber et al. 2007; Scheckhuber and Osiewacz 2008; van Diepeningen et al. 2010).

The second active line or research is that of prion biology. Indeed, an unusual case of inheritance discovered 70 years ago (Beisson-Schecroun 1962; Rizet and Delannoy 1950) is now demonstrated to be due to the prion properties of the Het-s protein involved in vegetative incompatibility (Coustou et al. 1997; Maddelein et al. 2002; Seuring et al. 2012). Because the Het-s proved to be more tractable than the other prion proteins, it has been used to decipher the structural determinants of prion formation (Greenwald et al. 2010; Ritter et al. 2005; Wasmer et al. 2008). It

has also enabled to clarify the role of prions. On the one hand, the Het-s prion acts as a detrimental selfish element during meiosis (Dalstra et al. 2003), and on the other hand, it is involved in the incompatibility reaction which prevents the spreading of deleterious genetic elements and is thus maintained at a balanced level in natural populations (Debets et al. 2012).

Third, developmental processes accompanying the life cycle, including ascospore ejection (Yafetto et al. 2008) and germination (Lambou et al. 2008; Malagnac et al. 2004), hypha differentiation (Brun et al. 2009) and fruiting body formation (Coppin et al. 2012; Espagne et al. 2011; Grognet et al. 2012; Jamet-Vierny et al. 2007; Malagnac et al. 2004; Peraza-Reyes et al. 2011; Silar 2011) are intensely studied in *P. anserina*. More integrated behaviours of the mycelium are also under investigation. These include the mechanisms of cell death during the vegetative incompatibility reaction (Pinan-Lucarre et al. 2007; Seuring et al. 2012) and crippled growth, an epigenetic cell degeneration caused by a non-conventional hereditary unit based on MAP kinases (Haedens et al. 2005; Kicka et al. 2006; Lalucque et al. 2012; Silar et al. 1999). The recognition phenomena that accompany these mechanisms are also under scrutiny: gamete recognition during fertilisation (Bidard et al. 2011; Coppin et al. 2005; Turgeon and Debuchy 2007), self-versus-non-self recognition during vegetative incompatibility (Chevanne et al. 2009, 2010; Paoletti et al. 2007) or interspecies recognition during Hyphal Interference (Paoletti and Saupe 2009; Silar 2005, 2012).

Although most of the phenomena presented above may appear restricted to *P. anserina* and closely related fungi, they are in fact archetypes of larger classes of processes that are universally present in all domains of life: How cells recognise each other? How cells die and how cells transmit information not directly encoded in their DNA to their daughter cells (epigenetic phenomenon)? How are the regulatory networks that regulate cell growth and differentiation wired? etc. There is no doubt that *P. anserina* still has much to offer as a convenient experimental model for research conducted on such topics. Moreover, because it is related to *N. crassa*, yet often presents contrasting characteristics, comparison between the two species is fruitful, especially if one seeks to encompass the behavioural diversity of fungi.

12.4.3 **P. anserina***: Its Gene Repertoire and Its Application in Biotechnology*

The *P. anserina* genome sequence has uncovered a plethora of genes encoding enzymes that in nature help the fungus to successfully invade its growth substrate and leave an abundant progeny, and some interestingly may have potential application in biotechnology.

Analysis of the CAZy repertoire showed that *P. anserina* has a large repertoire of enzymes involved in cellulose and xylan hydrolysis, but a smaller one for pectin

degradation (Espagne et al. 2008). *P. anserina* also lacks invertase and other GH32 enzymes and is unable to use saccharose or inulin as carbon source. This is in line with the fact that this fungus is rather late in the succession of fungi fruiting on dung: easy to digest carbon sources, such as saccharose, inulin and pectin, are likely no longer available, and *P. anserina* must be able to scavenge those that remain, i.e. xylan and cellulose. More unexpected was the discovery of many genes involved in lignin breakdown including 10 laccases/copper radical oxidases, 29 glucose/methanol/choline (GMC) oxidoreductases, 2 cellobiose dehydrogenases, 1 pyranose, 1 quinone, 1 copper radical and 1 galactose oxidase, 1 versatile peroxidase and 4 vanillyl-alcohol oxidases (Espagne et al. 2008; Poggeler 2011; P. Silar unpublished data). While the final proof of actual ligninolytic activity in *P. anserina* awaits the demonstration of mineralisation of lignin by chemical analysis, several arguments suggest that *P. anserina* is able to degrade lignin. Firstly, most of the enzymes harbour a secretion signal, suggesting that they act outside the cells. Secondly and interestingly, *P. anserina* is able to grow better on medium containing lignin as sole carbon source than on water agar (Espagne et al. 2008). Possibly, *P. anserina* is able to scavenge some usable carbon from lignin. Thirdly, it can complete its cycle on wood-derived materials, such as wood shavings or toothpicks! It is therefore able to efficiently degrade such food sources, possibly by partially removing lignin to gain access to the cellulose fibres. Other ascomycetes have demonstrated ligninolytic activities, including *P. chrysogenum* (Rodriguez et al. 1996), *Fusaria* (Lozovaya et al. 2006; Regalado et al. 1997) and especially many Xylariaceae, Sordariomycetes related to *P. anserina* common in decaying wood, which have a demonstrated brown rot ability (Pointing et al. 2003). It was recently established that brown rot (partial degradation of lignin) has evolved after white rot (complete degradation of lignin) in the Agaricomycetes (Floudas et al. 2012). The former rot being less energy consumptive than the latter is likely a more evolved strategy. Conceivably, coprophilous ascomycetes like *P. anserina* have evolved directly some rot akin to brown rot, as no ascomycete white rot is presently known.

In addition to producing enzymes, *P. anserina* differentiate dedicated hyphae resembling appressorium-like hyphae to penetrate the biomass (Brun et al. 2009), and the underlying signalling pathways are being explored (Lalucque et al. 2012). This permits a digestion from inside the material, which greatly accelerates the process (Brun et al. 2009) and likely increases recovery of energetic compounds to fuel reproduction (Malagnac et al. 2008). The availability of an efficient genetic analysis now permits to better define the modalities of biomass degradation in a model fungus, a feat not so easy to perform in Basidiomycetes in which gene inactivation is often not straightforward. For example, we recently genetically demonstrated the necessity of catalase for efficient lignin, but not cellulose, breakdown (Bourdais et al. 2012), likely to protect from the reactive oxygen species (ROS) associated with lignin depolymerisation. Intriguingly, inactivation of some catalase genes appears to increase the ability of the fungus to scavenge nutrient from wood, showing that a delicate balance in ROS production and elimination must be achieved to permit both efficient degradation and healthy growth and

reproduction. *P. anserina*, like *N. crassa* (Coradetti et al. 2012; Tian et al. 2009), is thus a good model to genetically study lignocellulose breakdown (Bey et al. 2013), especially with respect to the developmental processes involved in biomass penetration (Brun et al. 2009), which will have to be mastered if filamentous fungi are to be used in the future to directly transform waste products into valuable compounds. Moreover, its genes have been used to improve biomass saccharification by *Trichoderma* (Couturier et al. 2011; Turbe-Doan et al. 2012) and to produce vanillin (Hansen et al. 2009) or muconic acid (Curran et al. 2013), already showing exploitation in the bioenergy and food industries.

Another area in which *P. anserina* enzymes may be valuable is the remediation of polluted soils. Indeed, fungi with their peculiar lifestyle and their non-specific enzymes acting on plant biomass are likely in the future to be major organisms in pollution removal strategies (Harms et al. 2011; Silar et al. 2011). Often targeted fungi are basidiomycetes, because their enzymatic machinery appears more effective, or common soil ascomycetes such as Fusaria and to a lesser extent *Trichoderma*, as these seem to persist in soils (unlike the basidiomycetes). Preliminary experiments on the remediation of soils containing the toxic arylamine 3,4 dichloroaniline (DCA) indicate that *P. anserina* may efficiently inactivate this highly toxic pollutant by acetylation (Martins et al. 2009). It does so thanks to two acetyl transferases PaNat1 and PaNat2. PaNat2 appears to be the most important and is presently the enzyme that most efficiently acetylates DCA. Cosmopolitan coprophilous fungi may thus offer additional candidates for efficient removal of pollutants. What spectrum of molecules they are able to remove is yet unknown. However, the plethora of cytochrome P450 and oxidases encoded in the genome of *P. anserina* suggest that they may be able to detoxify a large spectrum of compounds. This is not unexpected as in their dung biotope they must be able to remove noxious metabolites secreted by competing bacteria and fungi, as well as those that may remain in the plant debris. Interestingly, these fungi are not known to generate disease in plant, animal or human and may be competitive in soils. They also often lack conidia and have short lifespans, which may prove valuable to limit spreading of the cleaning organism beyond the contaminated area.

The genome sequence revealed that *P. anserina* has the ability to produce many secondary metabolites. Indeed, it contains 18 polyketide synthases (PKS), 8 non-ribosomal peptide synthase (NRPS) and 3 enzymes with dual PKS/NRPS activity. It also has 2 terpene cyclases, 107 cytochromes P450 enzymes and a large array of oxidases acting on various putative substrates (Espagne et al. 2008). Among these, only the *PKS1* gene has been characterised (Coppin and Silar 2007). It is involved in the production of melanin, and its inactivation causes lack of pigment production at all stages of the life cycle. As in other fungi, these enzymes are encoded by gene clusters, one of which results from a horizontal transfer, likely from some *Aspergillus* (Slot and Rokas 2011). This cluster should confer the ability to produce sterigmatocystin. What metabolites are produced by these clusters is presently unknown, nor are the conditions for their expression. However, molecules with larvicidal activity against mosquito larvae, including sterigmatocystin, secosterigmatocystin and 13-hydroxyversicolorin, were purified

from an endophytic *Podospora sp.* closely related, if not identical, to *P. anserina* (Matasyoh et al. 2011). The role of these secondary metabolites in the physiology of the fungus is unknown. However, it is acknowledged that dung is a highly competitive biotope. Possibly, secondary metabolites are part of the repertoire that enables *P. anserina* to fight against other microorganisms for its place in the dung or to repel small animals such as nematodes, mites and collembola that may feed on it. Note that our *P. anserina* cultures are seldom attacked by mites, whereas we frequently find them on our plate cultures for other species, suggesting that *P. anserina* mycelium may not be very palatable!

Secondary metabolites are not the only tools that *P. anserina* uses to win competition. Indeed, it is able to kill other fungi by simple contact, a phenomenon called Hyphal Interference [see Silar (2012), for a review]. In addition to directly eliminating the competition, Hyphal Interference may also provide nitrogen, as dead hyphae contain chitin, a nitrogen-rich polymer. Intriguingly, *P. anserina* possesses an above average number of chitinases that could do the job (Espagne et al. 2008). The way Hyphal Interference operates is not clear and the potential fungicidal compounds are unknown. Interestingly, a MAP kinase pathway and an NADPH oxidase are crucial for this phenomenon, which involves a self-versus-non-self discrimination as observed in the immune systems of both plants and animals. Is it an ancient phenomenon already present in the ancestral eukaryotes or the result of some more recent convergent evolution? Only further studies in *P. anserina* and the other fungi exerting Hyphal Interference will tell. These researches may also end up with new sources of potent and safe antifungal compounds.

The final aspect for which *P. anserina* may be interesting is as a model to study appressorium biology. Indeed, not only does *P. anserina* differentiate some appressorium-like structures as stated above, but the germination of its ascospore appears to use the same machinery as the one used by true appressoria to penetrate living plants (Lambou et al. 2008). *P. anserina* thus offers an alternative to study this developmental process, especially to define the genes involved. Mutations affecting the germination have already been selected, and determination of the affected genes is underway (P. Silar et al. unpublished data).

12.5 Perspectives

With efficient systems for forward and reverse genetics in hand, development should now focus on establishing convenient tools for cytology and biochemical analyses. We have at our disposal strains with various organelles tagged with GFP: ribosomes (Lalucque and Silar 2000), peroxisomes (Ruprich-Robert et al. 2002), vacuoles (Pinan-Lucarre et al. 2005) and mitochondria (Sellem et al. 2007). New strains with nuclei, endoplasmic reticulum and cytoskeletal elements tagged with various fluorescent proteins are currently being constructed. Proteomic analysis has identified protein changes during senescence (Groebe et al. 2007). Similar

technology should be applied to identify changes during the various stages of the life cycle, which would complement the microarray data for the transcriptome. An inducible promoter, in addition to the strong promoters already available, may also come in handy, as at the present time none is available. The problem appears to be to find one that is truly off, as in our hands we always observed some leakiness of the promoters.

With the genome sequence and the new resulting tools, research with *P. anserina* appears still competitive. The size of the community working with this model is still small, so tools developed for more popular models, such as the complete deletion collection available for *N. crassa*, remain a long way ahead. Nevertheless, *P. anserina* may be considered as a "niche" fungus, in which peculiar and interesting phenomena with potential broad implication can be studied conveniently.

Acknowledgement I would like to thank all members of the Podospora community, especially from the Parisian area, for freely sharing information.

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Chapter 13
Recent Advances on the Genomics of Litter- and Soil-Inhabiting Agaricomycetes

Phil Kersten and Dan Cullen

13.1 Introduction

Woody biomass makes up the major portion of terrestrial carbon, and forest ecosystems contain enormous reservoirs of lignocellulose belowground, in dead trees, and litter. Decomposition of this recalcitrant material and mobilization of nutrients are essential for forest health [reviewed by Boddy and Watkinson (1995)]. Although mechanisms are incompletely understood, initial decomposition of lignocellulose is efficiently carried out by certain filamentous fungi, and the genomes of representative species have been recently sequenced. This review covers these genome studies and the insight they provide regarding lignocellulose degradation. Emphasis is placed on extracellular oxidative systems which are widely thought to be involved in lignin degradation but increasingly implicated in the depolymerization of cellulose and hemicellulose. Areas of uncertainty are highlighted. Detailed descriptions of the voluminous literature are not provided. Instead, interested readers are referred to earlier reviews (Cullen and Kersten 2004; Hatakka and Hammel 2010; Kersten and Cullen 2007).

13.2 Microbiology of Woody Litter Decay

Wood cell walls represent a complex and formidable substrate. *Cellulose*, essentially linear chains of β-1,4-linked cellobiose organized into microfibrils, is the major component. Where chains are tightly stacked, the polymer is crystalline and resistant to hydrolysis. Nevertheless, many microbes are capable of cellulose utilization by hydrolyzing the β-1,4 linkages [reviewed by Baldrian and Valaskova

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_13,
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(2008)]. *Hemicellulose* is also widely utilized although a more diverse set of hydrolases are needed to fully degrade the branched polymer [reviewed by van den Brink and de Vries (2011)]. In contrast to cellulose and hemicellulose, *lignin* is a complex phenylpropanoid polymer (Higuchi 1990; Ralph et al. 2004). Few microbes have the capacity to depolymerize lignin, and none have been convincingly shown to utilize native lignin as a sole carbon source (Hatakka and Hammel 2010).

Efficient wood degradation is typically attributed to Agaricomycetes, and two basic forms are recognized: white rot and brown rot. *White rot* fungi depolymerize and mineralize all cell wall components including cellulose, hemicellulose, and the more recalcitrant lignin. Decay patterns vary among fungal species and strains as well as among wood species, morphology, and composition (Blanchette 1991; Daniel 1994; Eriksson et al. 1990; Schwarze 2007). Cell wall erosion by most white rot fungi, including "*Phanerochaete chrysosporium*", involves simultaneous degradation of all three polymers, whereas *Ceriporiopsis subvermispora* selectively degrades lignin in advance of cellulose and hemicellulose (Blanchette et al. 1992, 1997; Srebotnik and Messner 1994). In this context, it should be noted that reports of lignin depolymerization should be suspect in the absence of persuasive experimental support such as cleavage of non-phenolic lignin model compounds (below) and the degradation of radiolabeled lignin or synthetic lignins. Experiments relying on commercially available "lignin" should be carefully interpreted as these preparations typically contain contaminants and sulfonated lignin of varying molecular weight.

In contrast to white rot, *brown rot* fungi modify lignin but the polymeric residue remains (Niemenmaa et al. 2007; Yelle et al. 2008, 2011). Also distinctive, brown rot fungi rapidly depolymerize cellulose (Gilbertson 1981; Kirk et al. 1991; Kleman-Leyer et al. 1992; Worrall et al. 1997) in advance of extensive weight loss. This observation, together with microscopic localization of decay and studies of cell wall porosity, strongly argue for the involvement of small molecular weight oxidants diffusing into cell walls (Blanchette et al. 1997; Cowling 1961; Flournoy et al. 1993; Srebotnik and Messner 1991; Srebotnik et al. 1988). Hydroxyl radical has been repeatedly implicated as the diffusible oxidant, and its production attributed to *Fenton* reactions ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \cdot OH$) (Arantes et al. 2011; Cohen et al. 2002, 2004; Xu and Goodell 2001). Typically invoked to explain brown rot, such reactive oxygen species may also be operative in white rot as recently suggested (Arantes et al. 2011; Gomez-Toribio et al. 2009). In any case, the role of hydroxyl radical in situ is unresolved, and any reasonable model must accommodate the generation of highly reactive radical at or near the substrate as well as the need for a plausible redox system [reviewed by Arantes et al. (2012) and Goodell (2003)]. The involvement of small molecular weight iron chelators (Xu and Goodell 2001), cellobiose dehydrogenase (Henriksson et al. 2000a, b), and hydroquinone redox cycling (Paszczynski et al. 1999; Suzuki et al. 2006) has been proposed.

Beyond the wood-decay Agaricomycetes, several litter-inhabiting fungi have the capacity to degrade lignin, albeit less efficiently. These include the commercial

button mushroom *Agaricus bisporus* (Durrant et al. 1991), other basidiomycetes and a few higher ascomycetes [reviewed by Eriksson et al. (1990) and Hatakka (2001)]. Certain litter-decomposing fungi likely play a crucial role in the transformation and degradation of humic substances, a major fraction of soil organic matter (Kluczek-Turpeinen et al. 2005; Snajdr et al. 2010; Steffen et al. 2002). Recently, a white rot fungus *Trametes* sp. has been shown to degrade humic substances, and a Fenton-based mechanism implicated (Grinhut et al. 2011a, b).

Unexpectedly, recent studies have also connected hydroxyl radical-based degradation of humic substances by ectomycorrhizal (ECM) fungi. Ectomycorrhiza obtain carbon from plant hosts, but under some conditions, soil organic matter may be at least partially degraded (Baldrian 2009; Cullings and Courty 2009). As described below, scant ECM genome evidence (Martin et al. 2008; Vaario et al. 2012) supports a role for facultative saprotrophy, but transcriptome analyses, together with lignin structure determinations, suggest mechanisms by which soil organic extracts could be degraded by *Paxillus involutus* (Rineau et al. 2012).

13.3 Physiology and Genetics

13.3.1 Peroxidases

Peroxidases catalyze oxidations of diverse substrates with reduction of peroxide, which groups these enzymes under EC 1.11.x [donor: hydrogen peroxide oxidoreductase] in the NC-IUBMB system of nomenclature (Fleischmann et al. 2004). Beyond this description of chemical reaction, the classification of peroxidases based on protein properties is evolving as new enzymes are discovered and structural details are delineated indicating structure–function relationships. Not only may a single peroxidase have diverse substrates, but peroxidases of distinctly different structures may catalyze the same reaction. Furthermore, peroxidases may have different modes of oxidation while catalyzing the same net overall reaction. This presents significant challenges for classification of peroxidases into groups that adequately reflects function, protein structure, and phylogenetic relatedness (Hofrichter et al. 2010).

The first opportunity to classify a selection of secreted fungal peroxidases based on their crystal structure occurred in the early 1990s; *lignin peroxidase* (LiP), *manganese peroxidase* (MnP), and *Coprinus cinereus* peroxidase (CiP) were shown to be sufficiently similar in overall 3D structure and active site to group as Class II peroxidases, distinct from Class I intracellular peroxidases, and Class III secretory plant peroxidases (Welinder 1992). Versatile peroxidase (VP), more recently discovered, shows properties of both LiP and MnP and is likewise a Class II peroxidase. However, other newly discovered heme-thiolate peroxidases (HtPs) and the dye-decolorizing peroxidases (DyPs) are clearly distinct in sequence, protein structure, and catalysis, justifying establishment of HtP-like

and DyP-like peroxidase superfamilies, separate from the Class II peroxidases. These complexities of fungal peroxidases and differences between "new" and "classic" families have been recently reviewed (Hofrichter et al. 2010). Salient properties of peroxidases as they may relate to litter and soil ecosystems are briefly summarized here.

13.3.1.1 High Oxidation Potential Class II Peroxidases (LiP, MnP, and VP)

Three Class II peroxidases, the LiPs (systematic name 1,2-bis (3,4-dimethoxyphenyl)propane-1,3-diol:hydrogen-peroxide oxidoreductase; EC 1.11.1.14), VPs (systematic name reactive-black-5:hydrogen peroxide oxidoreductase; EC 1.11.1.16), and MnPs (systematic name Mn(II):hydrogen peroxide oxidoreductase; EC 1.11.1.13) are able to modify lignin and other recalcitrant aromatic molecules in part due to their high oxidation capacity, in contrast to low redox potential peroxidases (e.g., CiP and NoP, also Class II peroxidases; see following section). LiP and VP are the most powerful of the peroxidases with redox potentials of approximately 1.5 V and able to oxidize non-phenolic lignin model compounds directly by one electron (Kersten et al. 1985; Kirk et al. 1986; Miki et al. 1986). Another feature of both LiP and VP is an enzyme surface tryptophan that mediates oxidation through long-range electron transfer (LRET) enabling oxidation of larger sterically hindered non-phenolic substrates (Choinowski et al. 1999; Doyle et al. 1998).

Unlike LiPs and VPs, the MnPs do not have a conserved Trp at the enzyme surface and are not able to efficiently oxidize non-phenolic aromatics directly. Instead, MnPs have a conserved solvent-exposed Mn-binding site in the vicinity of a heme propionate and therefore are able to catalyze the oxidation of Mn^{2+} to Mn^{3+} in the presence of peroxide and suitable Mn^{3+} chelators (Sundaramoorthy et al. 1994; Wariishi et al. 1992). The VPs similarly have a Mn-binding site and therefore have hybrid characteristics of both LiPs and MnPs (Ruiz-Dueñas et al. 2009). A likely physiological Mn^{3+} chelator for the MnP- and VP-catalyzed reactions is oxalate, which is known to be secreted by these fungi (Kuan and Tien 1993). Therefore, one role of MnPs and VPs is thought to be generation of diffusible Mn^{3+} chelates, which can oxidize phenolics directly. A more complex role may also be possible via oxidation of the chelates, thus generating other oxidizing species such as superoxide and perhydroxyl radical, which initiate radical chain reactions (e.g., in the presence of lipids) to generate ligninolytic radicals (Kapich et al. 1999). Phylogenetic analyses show the rise to two groups of MnPs, the so-called long-MnPs and the short-type hybrid MnPs which are more closely related to LiPs and VPs (Lundell et al. 2010).

The oxidizing capacity of these Class II peroxidases is achieved through the classical peroxidative cycle where "resting" enzyme is oxidized with peroxide by two electrons to generate Compound I enzyme intermediate. Compound I oxidizes substrates by one electron to produce oxidized substrate and Compound II enzyme

intermediate. The Compound II also oxidizes substrate, returning the enzyme back to "resting" state. In the case of LiPs and VPs with non-phenolic aromatics, the oxidized substrate are cation radicals and, as demonstrated with various substrates, the fate of the cation radical substrate intermediates is in large part determined by the substrate structure. Importantly, these reactions result in fragmentation of lignin model compounds and lignin (Miki et al. 1986; Tien and Kirk 1983).

Genes encoding Class II peroxidases have been identified in all lignin-degrading fungi, but not in brown rot or ECM. *Trametes versicolor* and *P. chrysosporium* genomes each feature ten LiP genes, but the corresponding proteins were not identified after 5 days growth in media containing ground aspen as sole carbon source (Table 13.1). On the other hand, ground pine wood and nutrient limited defined media support high transcript levels and secretion of several *P. chrysosporium* LiP and MnP isozymes (Vanden Wymelenberg et al. 2005, 2006b, 2009, 2010, 2011). Relative to LiPs, MnP-encoding genes appear more widely distributed. Sixteen MnP genes were identified in the genome of white rot fungus *Fomitiporia mediterranea*, and the corresponding proteins of seven are secreted in aspen cultures. Two *A. bisporus* MnP genes have been identified, and significant transcript accumulation occurs for one of these in compost (Morin et al. 2012). No Class II peroxidases were detected in the *Schizophyllum commune* genome. Although often classified as a white rot fungus and thereby presumed ligninolytic, the genetic repertoire of *S. commune* is consistent with weak or nonexistent lignin degradation (Boyle et al. 1992; Schmidt and Liese 1980).

Many wood- and litter-inhabiting fungi are clearly able to transform lignin or structurally related components of humic substances, but little is known about the role, if any, of high oxidation potential peroxidases in litter and soils. ^{14}C-labeled lignin and humic acid are degraded by litter decomposers *Gymnopus erythropus* and *Hypholoma fasciculare* when cultured on sterile leaf litter. MnP activity was higher in *G. erythropus* colonized litter, but degradation was less efficient for both species on non-sterile material, presumably due to interspecific competition (Snajdr et al. 2010). Similarly, laboratory experiments demonstrated degradation of ^{14}C-labeled humic acid and production of MnP by the litter decomposer, *Collybia dryophila* (Steffen et al. 2002). Laboratory studies of *P. chrysosporium*-colonized wood chips and soil have quantified transcript levels of specific LiP and MnP genes (Bogan et al. 1996b; Janse et al. 1998), and MnP activity was measured in organopollutant-contaminated soil (Bogan et al. 1996a).

13.3.1.2 Low Oxidation Potential Class II Peroxidases (CiP and NoP)

"*Coprinopsis cinerea* peroxidase" or CiP (systematic name phenolic donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7) is not able to oxidize non-phenolics such as veratryl alcohol; neither does it have the manganese-binding site of VP or MnP. Rather, CiP very efficiently oxidizes phenolics as a low oxidation peroxidase. As with other Class II peroxidases, CiP is similar in structure with conserved proximal and distal histidines near the heme active site; however, when compared

Table 13.1 Agaricomycete genes potentially involved in lignocellulose degradation: mass spectrometry-identified proteins—gene number

	White rot										Brown rot						Saprotrophs		ECM
Gene	Ad	Cs	Ds	Fm	Ha	Pc	Ps	Sc	Sh	Tv	Cp	Fp	Gt	Pp	Sl	Wc	Ab	Cc	Lb
LiP	0-0	0-0	0-0	0-0	0	0-10	0-0	0	0-0	0-10	0-0	0-0	0-0	0-0	0-0	0-0	0-0	0	0-0
MnP	0-5	2-13	0-9	7-16	8	0-5	1-10	0	0-10	2-13	0-0	0-0	0-0	0-0	0-0	0-0	2	0	0-0
VP	0-0	0-2	0-3	0-0	0	0-0	0-0	0	0-0	1-2	0-0	0-0	0-0	0-0	0-0	0-0	0	0	0-0
HtP	1-16	0-9	0-4	0-4	5	0-3	0-8	3	0-10	0-3	0-2	1-4	0-6	0-5	0-3	0-5	24	8	0-5
DyP	1-11	0-0	1-1	0-3	1	0-0	1-5	0	0-2	1-2	0-0	0-0	0-0	0-2	0-0	0-0	0	4	0-2
Lac	0-7	1-7	3-11	5-10	13	0-0	2-12	2	0-15	4-7	0-6	0-5	4	0-3	1-4	1-5	12	17	0-9
GLX	0-2	0-0	0-5	0-0	0	0-1	1-3	0	0-3	1-5	0-0	0-0	0-0	0-0	0-0	0-0	3	0	0-0
Cro1	0-0	0-0	0-1	0-1	1	0-1	0-0	0	0-0	0-1	0-1	0-1	0-0	0-0	0-0	0-1	2	3	1-6
Cro2	2-3	0-2	1-1	0-1	1	0-2	1-3	1	1-3	1-1	3-4	1-1	2-1	0-1	0-1	1-1	2	2	2-2
Cro3/5	1-1	0-1	1-1	0-1	1	0-3	1-1	0	0-1	1-1	0-0	0-1	0-0	1-1	0-1	1-1	1	1	0-2
Cro6	1-2	0-1	0-1	0-1	1	0-1	0-1	1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	1	1	0-0
CDH	0-1	0-1	1-1	0-1	1	0-1	1-1	1	1-1	1-1	1-1	0-0	0-1	0-0	0-2	0-0	1	1	0-0
GH61	1-19	0-9	3-15	0-13	10	2-15	6-13	22	1-16	1-16	0-10	0-4	1-4	0-2	0-5	0-2	11	35	0-5
GH6	2-2	1-1	1-1	0-2	1	1-1	1-1	1	1-1	1-1	0-2	0-0	0-0	0-0	0-1	0-0	1	5	0-0
GH7	2-6	1-3	1-4	0-2	1	3-6	1-5	2	1-3	1-4	0-2	0-0	0-0	0-0	0-0	0-0	1	6	0-0
UP	56	98	43	20	NA	27	30	NA	36	44	49	65	53	9	NA	21	NA	NA	NA
TP	151	121	180	85	NA	90	135	NA	208	218	269	253	174	64	NA	171	NA	NA	NA

Hyphenated entries report the number of published proteins identified by mass spectrometry followed by the total number of predicted genes. Citations and abbreviations: (Floudas et al. 2012) Ad, *Auricularia delicata*; Ds, *Dichomitus squalens*; Fm, *Fomitiporia mediterranea*; Ps, *Punctularia strigosozonata*; Sh, *Stereum hirsutum*; Tv, *Trametes versicolor*; Cp, *Coniophora puteana*; Fp, *Fomitopsis pinicola*; Gt, *Gloeophyllum trabeum*; and Sc, *Wolfiporia cocos*. (Fernandez-Fueyo et al. 2012) Cs, *Ceriporiopsis subvermispora*. (Olson et al. 2012) Ha, *Heterobasidion annosum*. (Vanden Wymelenberg et al. 2011) Pc, *Phanerochaete chrysosporium*. (Ohm et al. 2010) *Sc*, *Schizophyllum commune*. (Martinez et al. 2009) Pp, *Postia placenta*. (Eastwood et al. 2011) Sl, *Serpula lacrymans*. (Morin et al. 2012) Ab, *Agaricus bisporus*. (Stajich et al. 2010) Cc, *Coprinopsis cinereus*. (Vincent et al. 2012) Lb, *Laccaria bicolor*. The total number of proteins identified in aspen-containing medium and those designated as uncharacterized are indicated by TP and UP, respectively. Additional abbreviations include the following: LiP, lignin peroxidase; MnP, manganese peroxidase; VP, versatile peroxidase; HTP, heme-thiol peroxidase; DyP, dye-decolorizing peroxidases; Lac, laccase; GLX, the copper radical oxidase glyoxal oxidase; CROs, copper radical oxidases most closely related to those of *P. chrysosporium* (Vanden Wymelenberg et al. 2006a); CDH, cellobiose dehydrogenase; and GH61, GH6, and GH7, members of the glycoside hydrolase families 61, 6, and 7, respectively

to LiP, the distal side substrate channel is more open to easily oxidized reducing aromatic substrates (Kunishima et al. 1994; Petersen et al. 1994). However, CiP can be engineered to have activity with veratryl alcohol, the standard high redox potential substrate for LiP, by mutations to introduce the surface Trp and negatively charged microenvironment (Smith et al. 2009). Based on sequence analysis, genes encoding such low oxidation peroxidases have also been identified in brown rot fungi *F. pinicola*, *P. placenta*, and *W. cocos* as well as the white rot fungi *C. subvermispora*, *C. strigosozonata*, *F. mediterranea*, *H. annosum*, *S. hirsutum* (Floudas et al. 2012), and *P. chrysosporium*. Designated NoP, the *P. chrysosporium* structure has been studied in some detail (Larrondo et al. 2005). High transcript levels and secreted proteins have not been observed, and the role of these peroxidases remains uncertain.

13.3.1.3 Heme-thiolate Peroxidases

Heme-thiolate peroxidases (HtPs) comprise a superfamily of secreted fungal peroxidases distinguished from the Class II peroxidases, not only by the proximal cysteine and distal glutamate heme ligands but also by distinctive 3D structure, protein sequence, and remarkable catalytic capacity. HtPs are classified based on dominating reactions that are consistent with *chloroperoxidase* (CPOs; systematic name chloride:hydrogen-peroxide oxidoreductase; EC 1.11.1.10) and aromatic peroxygenase (APOs; systematic name substrate:hydrogen peroxide oxidoreductase (RH-hydroxylating or -epoxidizing); EC 1.11.2.1) activities.

CPO from *Caldariomyces fumago* halogenates suitable organic substrates in the presence of peroxide and halide (Morris and Hager 1966; Shaw and Hager 1959). The primary function of CPO here is oxidization of chloride (Cl^{-}) to hypochlorous acid, which is a strong oxidant and able to chlorinate the organic compounds (Murali Manoj 2006). Bromide and iodide are also oxidized by CPO, but not fluoride. If halide is absent, CPO oxidizes suitably substituted phenols and anilines directly. In the case of chlorinated phenols, oxidations with CPO are much more efficient than with horseradish peroxidase, LiP or VP (Casella et al. 1994; Longoria et al. 2008; Osborne et al. 2007). Besides halogenation and phenol oxidation capability, CPO also has peroxygenase properties, resembling cytochrome P450-dependent monooxygenase (also a heme-thiolate) in catalysis and structure (Manoj and Hager 2008; Sundaramoorthy et al. 1995). CPO is able to epoxidize alkenes and hydroxylates benzylic carbons via a peroxygenase mechanism (Manoj and Hager 2008), but oxygen transfer to less-activated molecules such as alkanes or aromatic rings is not catalyzed. The proposed functional roles of the enzyme are varied, from biosynthesis of chlorinated metabolites (Morris and Hager 1966) to antimicrobial activity, due to the biocidal activity of hypochlorite (Bengtson et al. 2009). A consequence of the nonspecificity of CPOs and the reactive chemical species generated is the chlorination of lignin (Ortiz-Bermúdez et al. 2007).

In contrast to CPO, the fungal *peroxygenases* (APOs) from *Agrocybe aegerita* (AaeAPO; Ullrich et al. 2004), *Coprinellus radians* (CraAPO; Anh et al. 2007;

Aranda et al. 2009), and *Marasmius rotula* (MroAPO; Gröbe et al. 2011) are all able to catalyze oxygen transfer reactions to aromatic rings, in addition to other divers reactions. AaeAPO was first described as a haloperoxidase but with further characterization shown to be a functional hybrid with properties of both a peroxidase and a monooxygenase (Hofrichter et al. 2010). APOs have a wide variety of substrates including polyaromatics (e.g., naphthalene), recalcitrant heterocycles (e.g., pyridine), ethers (with *O*-dealkylation resulting), and alkanes (e.g., propane and hexane). The chemical transformations include hydroxylation, epoxidation, N-oxidation, sulfooxidation, bromination, and one-electron oxidations. However, the array of reactions is not shared by all APOs; the MroAPO of *M. rotula* does not have the brominating activity of AaeAPO and CraAPO (Gröbe et al. 2011).

All sequenced Agaricomycete genomes feature genes encoding HtPs, but their number and expression vary substantially. Peptides were identified in culture filtrates of *A. delicata* and *F. pinicola* (Floudas et al. 2012). LC-MS/MS could not detect HTPs in *P. chrysosporium* or *P. placenta* cultures (Vanden Wymelenberg et al. 2011), although each species exhibited significant transcript accumulation of two HtP genes in wood-containing cultures relative to glucose medium. More impressive, 24 HtP genes were predicted in the *A. bisporus* genome, and 16 of these were significantly upregulated in compost (Morin et al. 2012). This observation suggests that HtPs may play an important role in metabolism of partially decomposed litter and humic substances. A Dacrymycete classified as a brown rotter, *Dacryopinax* sp., contains six putative HtP-encoding genes, and the corresponding protein has been detected for two of these (Floudas et al. 2012).

13.3.1.4 Dye-Decolorizing Peroxidases

Prototypical DyP was isolated from the Agaricomycete *Bjerkandera adusta* [first reported as *Geotrichum candidum* Dec 1, re-identified as *Thanatephorus cucumeris* Dec 1, and then *B. adusta* (Ruiz-Dueñas et al. 2011)] because of its dye-decolorizing activities (Kim and Shoda 1999; Kim et al. 1995; Sugano 2009). Subsequent structural and sequence comparisons indicate that the peroxidase is unlike any previously characterized peroxidase (Sugano et al. 2007; Zubieta et al. 2007). DyPs (systematic name Reactive-Blue-5:hydrogen peroxide oxidoreductase; EC 1.11.1.19) have a proximal histidine, but unlike the Class II and HtP peroxidases, DyPs have a distal Asp. The model substrate, Reactive Blue 5, is converted to products by a combination of oxidation and hydrolytic steps. Other dyes, anthraquinone derivatives and typical peroxidase substrates, are also oxidized. Structural and biochemical characterization of DyP-like peroxidases from bacteria is also reported (Brown et al. 2012; Zubieta et al. 2007). In the case of *Amycolatopsis*, the DyP2 appears multifunctional, showing high peroxidase activity, manganese peroxidase activity, and also a mode of oxidase activity with 4-methoxymandelic acid (Brown et al. 2012). Liers et al. (2013) have recently compared activities of fungal DyP with heme peroxidases, and considering the

complex catalytic properties of the DyPs, the physiological roles are most likely diverse.

The number of DyP-encoding genes varies significantly among Agaricomycetes. Certain efficient lignin degraders such as *P. chrysosporium* and *C. subvermispora* have none, while 11 genes are predicted in the *A. delicata* genome (Table 13.1). Of eight white rot cultures subjected to LC-MS/MS analysis, four contained the corresponding peptides. Five brown rot fungi have no DyP genes. Only one brown rot fungus, *P. placenta*, is predicted to have DyP genes, but the corresponding proteins were not detected in aspen-containing medium (Vanden Wymelenberg et al. 2011). Significant protein levels occur in ground wood culture filtrates of *T. versicolor*, *D. squalens*, *A. delicata*, and *P. strigozonata* where a single DyP constituted 2.2 %, 1.3 %, 0.1 %, and 0.08 %, respectively, of the total spectra (Floudas et al. 2012).

13.3.2 Extracellular Peroxide Generation

It is readily apparent that extracellular peroxide is a key player in lignocellulose biotransformation in view of the importance of peroxide-dependent peroxidases secreted by fungi (previous section). Even prior to the discovery of these peroxidases, a correlation was observed between peroxide production and the physiology of ligninolysis by *P. chrysosporium* (Faison and Kirk 1983; Forney et al. 1982; Keyser et al. 1978). There appears to be several possible mechanisms for peroxide production, including by MnP with fungal metabolites oxalate and glyoxylate; peroxide is evidently generated as a consequence of reactions of oxygen with carbon-centered radical substrate intermediates (Kuan and Tien 1993; Urzúa et al. 1998). Oxidases that catalyze reduction of oxygen to peroxide, and where evidence indicates an extracellular role in supporting peroxidase activities, are briefly summarized here.

13.3.2.1 Copper Radical Oxidases

Glyoxal oxidase (GLX) was first reported in cultures of *P. chrysosporium* where its activity was correlated with substrates glyoxal and methylglyoxal in culture, and with LiP activity (Kersten and Kirk 1987). Importantly, the activity of GLX was activated by interaction with LiP (Kersten 1990). Sequence comparisons and spectroscopic comparisons indicate that GLX has a similar active site to that of the copper radical oxidase (CRO) galactose oxidase (Kersten and Cullen 1993; Whittaker et al. 1996, 1999). Copper radical oxidases have two one-electron acceptors: a copper (II) metal at the center of the active site and a Cys-Tyr radical forming a metallo-radical complex (Whittaker 2005). Alignment of galactose oxidase with GLX indicates that the catalytic domain of GLX supplies copper ligands Tyr135 Tyr377 and His378 while Cys70 is cross-linked with Tyr135 to

form an internal radical cofactor of the sevenfold β-barrel domain (also described as a super-barrel or β-flower). The C-terminal domain supplies His471 copper ligand on a loop through the center of the catalytic barrel. Analysis of the *P. chrysosporium* genome indicates multiple CROs where predicted mature protein sequences diverge substantially from one another, but the residues coordinating copper and constituting the radical redox site are conserved (Vanden Wymelenberg et al. 2006a).

More recent analysis of Agaricomycete genomes has revealed widespread distribution of GLX and related *copper radical oxidases* (Table 13.1). GLX homologs are lacking from brown rot fungi, *C. cinereus* and *L. bicolor*, but present in most lignin-degrading fungi. Notable exceptions include *C. subvermispora*, a selective and highly efficient lignin degrader. Possibly, the related copper radical oxidases *cro2*, *cro3*, and *cro6* compensate by oxidizing an array of metabolites unique to *C. subvermispora* decay. Along these lines, elevated transcript levels of the *C. subvermispora cro2* gene were observed in wood-containing medium, and peptides corresponding to CRO5 were detected in medium using microcrystalline cellulose as sole carbon source (Fernandez-Fueyo et al. 2012). Earlier reports showed that the substrate preference of *P. chrysosporium* CRO2 differed sharply from GLX (Vanden Wymelenberg et al. 2006a).

13.3.2.2 GMC Oxidoreductases

Another oxidase produced by *P. chrysosporium* and a select number of other white and brown rot fungi is *pyranose 2-oxidase* (P2O), which oxidizes glucose at C-2 to produce D-*arabino*-hexos-2-ulose (glucosone) with reduction of oxygen to peroxide (Baute and Baute 1984; de Koker et al. 2004; Dietrich and Crooks 2009; Giffhorn 2000). The protein is a large homotetrameric flavoprotein with a subunit size of about 70 kDa (Hallberg et al. 2004). It is a member of the glucose-methanol-choline oxidoreductase family (Albrecht and Lengauer 2003), a superfamily of proteins including *Drosophila melanogaster* glucose dehydrogenase, *Aspergillus niger* glucose oxidase, *Hansenula polymorpha* methanol oxidase, and *Escherichia coli* choline dehydrogenase (Cavener 1992). The periplasmic and extracellular distribution of pyranose 2-oxidase in wood decayed by *P. chrysosporium* is consistent with that of MnP, suggesting a role in extracellular peroxide generation (Daniel et al. 1994). An alternative role for pyranose 2-oxidase is the synthesis of the antibiotic cortalcerone (Koths et al. 1992), but many fungi that have pyranose 2-oxidase do not have aldo-2-ulose dehydratase required for cortalcerone synthesis (Baute and Baute 1984). Quinones are alternate electron acceptors in place of oxygen, and therefore, the oxidase may have a role in redox cycling during lignocellulose degradation (Pisanelli et al. 2009). P2O-encoding genes are predicted in the genomes of brown rot fungi *G. trabeum* and *S. lacrymans* as well

as the white rot fungi *A. delicata*, *P. strigosozonata*, *T. versicolor*, and *P. chrysosporium*.

Another GMC oxidoreductase involved in peroxide generation is the extracellular monomeric *aryl-alcohol oxidase* (AAO) [recently reviewed by Hernández-Ortega et al. (2012)]. AAO substrates may be both lignin-derived metabolites, as well as aromatic fungal metabolites synthesized de novo (de Jong et al. 1994; Gutiérrez et al. 1994). The aromatic alcohol substrates are oxidized to the corresponding aldehydes by AAOs, and these aldehydes reduced back to alcohols by intracellular aryl-alcohol dehydrogenases, thus establishing a redox cycle for the generation of extracellular peroxide using reducing equivalents derived from intracellular metabolism. A possible role for AAO in preventing polymerization of lignin fragments by reduction of quinone and phenoxyradicals is also described (Marzullo et al. 1995). The AAO from *P. eryngii* has been heterologously expressed in *E. coli* (Ruiz-Dueñas et al. 2006) and crystal structure determined (Fernandez et al. 2009). The GMC oxidoreductase *methanol oxidase* is also proposed to have a role in peroxide production using methanol released from lignin methoxyls (Nishida and Eriksson 1987). Although a signal peptide is not evident from gene structure, the oxidase appears to have an extracellular role in wood decay with *G. trabeum* (Daniel et al. 2007). Putative methanol oxidase- and AAO-encoding genes have been identified in a wide range of white rot and brown rot fungi (Hernández-Ortega et al. 2012). In *C. subvermispora* and *P. chrysosporium*, the AAO genes show no transcript accumulation in medium containing ground wood relative to glucose-containing media (Fernandez-Fueyo et al. 2012; Vanden Wymelenberg et al. 2011).

A potentially important oxidoreductase, *cellobiose dehydrogenase* (CDH), oxidizes cellodextrins, mannodextrins, and lactose. In addition to the dehydrogenase, the mature protein contains a heme prosthetic group and a cellulose-binding module (Hallberg et al. 2000). Electron acceptors include quinones, phenoxyradicals, and Fe^{3+}, and involvement in hydroxyl radical generation has been proposed. CDH genes are widely distributed (Table 13.1) as are "glycoside hydrolase" family 61 (GH61) genes. Recently shown to act as *copper-dependent monooxygenases* (Quinlan et al. 2011; Westereng et al. 2011), the GH61s can act together with CDHs to boost cellulose depolymerization (Harris et al. 2010; Langston et al. 2011). The precise roles(s) and interaction(s) between these genes remain to be clarified.

While the abovementioned oxidoreductases can be functionally categorized on the basis of sequence conservation, many variants defy simple classification. For example, peptides corresponding to *S. hirsutum* protein model #118344 (http://genome.jgi.doe.gov/Stehi1/Stehi1.home.html) constitute 5.3 % of the total spectra observed in wood-containing medium. The protein features a secretion signal and InterPro domains that point toward a GMC oxidoreductase, but little additional information allows a firm definition.

13.3.3 Laccases

Laccases are diverse in origin (plants, fungi, and bacteria) and properties [see review (Thurston 1994)]. Brief description of fungal laccase is presented here to highlight the essential properties distinguishing it from the enzymes in the foregoing sections. Laccase (systematic name benzenediol:oxygen oxidoreductase; EC 1.10.3.2) catalyzes the four-electron reduction of oxygen to water with the electrons derived by four one-electron oxidations of substrate (typically phenols or aryl amines). The four-electron reduction is achieved with four copper ions at three enzyme sites: the T1 site contains a type 1 copper in tight coordination with cysteine which gives laccase its blue color, the T2 site has a type 2 copper with characteristic EPR signal, and the T3 site has a pair of strongly coupled EPR-silent type 3 coppers (Bertrand et al. 2002). The T1 site mediates oxidations with transfer of electrons to the T2/T3 trinuclear center where electrons are transferred to oxygen. The capacity for single-electron oxidations by laccase from *T. versicolor*, in comparison with LiP and Class III HRP, was demonstrated with 1,2,4,5-tetramethoxybenzene producing the corresponding cation radical as immediate product (Kersten et al. 1990). Although the laccase oxidized this methoxybenzene congener, it did not have the same capacity as the peroxidases to oxidize methoxybenzenes of higher potential. Laccase oxidation of phenols generates intermediates which may undergo further enzyme-catalyzed oxidation (e.g., generating quinones) or the unstable intermediates may undergo nonenzymatic reactions such as polymerizations (Thurston 1994). Laccase genes are widely distributed among fungi (Table 13.1) but not essential for ligninolysis as demonstrated by the lack of the enzyme in *P. chrysosporium* (Martinez et al. 2004). Differential expression among paralogs is commonly observed (see, e.g., Castanera et al. 2012; Floudas et al. 2012), although the role of genetic multiplicity is poorly understood. Excluding *P. chrysosporium*, considerable evidence suggests that laccase may have a role in lignin modification or plant litter decay (Bourbonnais et al. 1997; Kellner et al. 2007).

13.3.4 Hypothetical and Uncharacterized Proteins

A persistent concern has been the incomplete understanding of predicted proteins lacking significant homology to those of known function (herein referred to as "hypothetical"), some of which are translated and secreted (herein referred to as "uncharacterized"). The dimensions of this issue are staggering. For example, 21 % of the 13,761 *C. puteana* protein models showed no significant similarity to NCBI NR database entries (Floudas et al. 2012). Mass spectrometry analysis of filtrates from aspen-containing media identified 269 separate proteins, and 49 of these were designated "uncharacterized" (Table 13.1). High expression levels, conserved domains, and/or structural features are sometimes observed for these

uncharacterized proteins. Thus, *C. puteana* protein #125481(http://genome.jgi.doe.gov/Conpu1/Conpu1.home.html) constitutes 1.9 % of total spectra and has a predicted secretion signal and an InterPro conserved domain of unknown function (DUF 1793).

13.4 Challenges and Future Prospects

The daunting number of hypothetical proteins and the lack of appropriate experimental tools present significant obstacles to progress in this area. Transcript and *secretome* profiles provide clues, but detailed functional analysis requires biochemical characterization of heterologously expressed proteins and/or targeted gene replacement/suppression. The latter goal has been particularly difficult to achieve because genetic transformation of filamentous basidiomycetes has typically involved low rates of homologous recombination. Recently, this obstacle has been overcome by isolating Ku knockouts that impair nonhomologous end joining in *S. commune* (de Jong et al. 2010), *C. cinereus* (Nakazawa et al. 2011), and *P. ostreatus* (Salame et al. 2012). Demonstrating the power of the approach, Salame et al. demonstrated the importance of *P. pleurotus* MnP4 by successfully inactivating the VP gene. Likely, this experimental approach will be applied to additional Agaricomycetes in the future.

Availability of increasing numbers of genome sequences facilitates high-throughput approaches for elucidating community structure and physiological processes in soils. In addition to the widely used ribosomal DNA and/or the internal transcribed spacer region (ITS) (Buee et al. 2009), the distribution of highly conserved genes such as those encoding laccases and cellobiohydrolases gauge fungal diversity in different soils and soil horizons (Baldrian et al. 2012; Luis et al. 2005). When combined with rRNA sequence, a more complete picture of microbial populations and active metabolism emerges. Degenerate primers amplified cDNAs corresponding to basidiomycete laccases, MnPs, and HtPs (Kellner et al. 2010), and more recent "metatranscriptomic" investigations have provided a more global view of transcript levels (Simon and Daniel 2011). Examples include the assessment of soil gene expression in response to phenanthrene contamination (de Menezes et al. 2012) and the measurements of transcript and populations in various forest soils (Damon et al. 2012). Both investigations identified transcripts corresponding to Agaricomycete degradative enzymes, and the latter study also employed 18S rRNA sequencing to assign broad taxonomic affiliations (Damon et al. 2012). *Metatranscriptome* approaches can be enhanced or extended to functional analysis by expression of full-length genes in *Saccharomyces cerevisiae* (Bailly et al. 2007; Damon et al. 2011; Kellner et al. 2011).

Also promising are the prospects for direct detection of fungal proteins and enzyme activities in natural substrates and/or field soils. Immobilization of fluorescent substrates allows visualization of hydrolytic enzymes on decaying litter, leaves, and wood (Baldrian and Vetrovsky 2012). In addition to such localization,

metabolite identification could play a key role in defining the processes involved in decomposition. Thus, NMR and GC/MS of field-collected decayed wood provided evidence for Fenton-based brown rot (Martinez et al. 2011). Fenton chemistry was also implicated in laboratory studies of *P. involutus* cultured on soil extracts using FTIR and GC/MS coupled to enzyme activities and to mRNAseq (Rineau et al. 2012). *Metaproteomics* [reviewed by Hettich et al. (2012)] offer unparalleled opportunities for understanding microbial processes as demonstrated by recent mass spectrometry of soil (Keiblinger et al. 2012) and leaf litter samples (Schneider et al. 2012). Although challenging technical issues remain, these experimental approaches are beginning to shed light on the roles and interactions of fungi in forest soils.

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Chapter 14
Archaeorhizomycetes: Patterns of Distribution and Abundance in Soil

Anna Rosling, Ina Timling, and D. Lee Taylor

14.1 Introduction

Archaeorhizomycetes represents one of the most ubiquitous lineages of soil fungi, and its formal description adds a prominent branch to the Taphrinomycotina among the basal Ascomycota (Rosling et al. 2011). Fungi in the class are strongly associated with soil environments containing plant roots. However, experimental analyses suggest that interactions with roots are neither mycorrhizal nor pathogenic. Instead, species in the Archaeorhizomycetes may exist along a continuum from root endophytic to free-living saprophytic life strategies. It is possible that Archaeorhizomycetes are mycoparasitic, but these life strategies have not yet been studied. Among thousands of published environmental sequences belonging to the class, only one was neither from soil nor roots. The sequence (GenBank Acc nr. EF67470) was cloned from samples of particulate organic matter collected in sediment from a freshwater stream (Bärlocher et al. 2008). While this could indicate that Archaeorhizomycetes is not restricted to terrestrial habitats, a more likely explanation is that the sequence originated from terrestrial material, i.e., spores, soil, and organic matter, that were deposited in the stream. Hence, all

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_14,
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available evidence supports the notion that Archaeorhizomycetes is restricted to vegetated terrestrial ecosystems (Porter et al. 2008; Rosling et al. 2011).

Based on similarity to environmental ITS and LSU sequences available in public databases, the class has been estimated to comprise more than 250 putative species (Rosling et al. 2011). Strong biogeographic patterns with significant global association between geographic and phylogenetic distance were detected across the class. Despite these strong biogeographic patterns, a number of putative species, such as *A. finlayi*, have a broad geographic distribution. Habitat specificity towards host species or genus, e.g. Tsuga, Picea, and Pinus, was detected for many putative species, while others have a broader habitat range (Rosling et al. 2011). It is possible that species that show habitat specificity are more closely associated with roots than species with broad distribution. It is important to keep in mind that Archaeorhizomycetes is an ancient class of fungi and may thus encompass diverse life strategies and ecologies.

In this chapter we analyze available ITS, LSU, and SSU sequences and their associated publications to discuss global distribution and abundance of Archaeorhizomycetes. Furthermore we expand our knowledge about Archaeorhizomycetes by analyzing publicly available ITS-LSU sequences from ten studies, seven with sequences in GenBank as well as unpublished sequence data from three studies: the North American Arctic Transect (NAAT) (Timling et al. unpublished), black spruce forest (TKN), and successional gradient in an upland ecosystem (UP) (Taylor et al. 2010).

14.1.1 Global Distribution

Based on new analysis of available environmental sequences of the ITS, LSU, and SSU regions, we demonstrate that species in the class Archaeorhizomycetes occur on all continents, except Antarctica, and in most terrestrial biomes, including tundra, taiga, tropical rainforest, temperate forest, and grasslands (Fig. 14.1). The size of dots in Fig. 14.1 illustrates the number of sites from which sequences of Archaeorhizomycetes have been identified in different regions. The number of observations is strongly biased towards regions where many studies of soil fungal communities are performed using molecular identification methods, i.e., Europe and North America. Ecosystems in these regions are mostly comprised of coniferous forests (Buscardo et al. 2010; Cox et al. 2010; Lindahl et al. 2007; Parrent and Vilgalys 2007; Rincon and Pueyo 2010; Taylor et al. 2008; Urban et al. 2008) and tundra-/shrub-type ecosystems (Bjorbaekmo et al. 2010; Bougoure et al. 2007; Deslippe et al. 2012; Schadt et al. 2003) but also include mixed deciduous forests (Edwards and Zak 2011; Stefani et al. 2009).

Observations of sequences belonging to the Archaeorhizomycetes in the Southern Hemisphere include the following: tropical rain forest in southwestern Costa Rica (Porter et al. 2008); tropical mountain pine forest in North Eastern Australia (Curlevski et al. 2010); dry sclerophyll forest in New South Wales, Australia (Chen

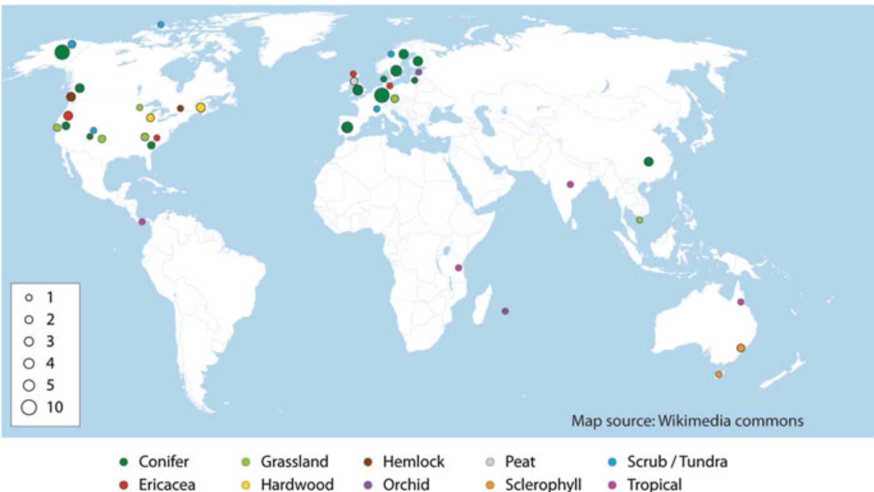


Fig. 14.1 Map of the world illustrating location and ecosystem where Archaeorhizomycetes has been detected using environmental sequencing. Size of *dots* corresponds to the number of sampling occasions (i.e., different sites or different studies from the same site) in which sequences of Archaeorhizomycetes have been detected. Data are compiled using sequences of the ITS, LSU, and SSU rRNA region available in GenBank with associated publications as well as additional sequences from three studies from the North American Boreal Forest and Arctic (Taylor unpublished data)

and Cairney 2002); wet sclerophyll forests in Tasmania (Tedersoo et al. 2009); subtropical pine forests in south central China (Huang et al. 2012); and in roots of the terrestrial orchid *Phaius pulchelus* collected on La Reunion, in the Pacific Ocean (Martos et al. 2012). Recent samples from unpublished studies in tropical regions add four more locations for Archaeorhizomycetes to the world map. These include samples collected from bulk soil associated with unhealthy *Axonopus compressus* (Blanket grass) in Singapore (HQ436085), root tips of *Allanblackia stuhlmannii* (a flowering tree indigenous to Tanzania) in the East Usambara Mountains in North Eastern Tanzania (unpublished data, Helena Ström, SLU Sweden), and brinjal (eggplant) rhizosphere soil in India (JQ989336) as well as one more tropical pine forest in Zhenjiang, China (HE814241) (Fig. 14.1). We expect that increased sampling and molecular identification of soil fungi in the Southern Hemisphere will increase the number of observations of Archaeorhizomycetes in tropical and subtropical ecosystems. The significant association between geographic and phylogenetic distance is notable in the global phylogeny of Archaeorhizomycetes where all Alaska sequences are found within the upper 2/3 of the tree among sequences derived mainly from boreal and coniferous ecosystems [Fig. 3 in Rosling et al. (2011)]. We thus expect new lineages, at the level of genus and families, to emerge in the phylogeny of Archaeorhizomycetes as sampling of the Southern Hemisphere proceeds.

Intensified studies of fungi in tropical ecosystems have generally resulted in the recognition of many previously unknown species (Hawksworth 2012). This pattern may become apparent for Archaeorhizomycetes as well, as more sequences are made publicly available from studies in tropical ecosystems. In a comparison of ectomycorrhizal-dominated boreal and tropical forests, McGuire and co-workers studied soil fungal communities by sequencing the ITS and LSU rRNA. The study is still unpublished, but sequences from the study are available in GenBank. While 13 % of the sequences from Delta Junction in Alaska belonged to Archaeorhizomycetes, none of the sequences from their tropical forest sites belonged to this class. This result, as well as the existing global patterns (Fig. 14.1), suggests that fungi in the Archaeorhizomycetes may be more abundant in boreal compared to tropical forest ecosystems.

14.1.2 Abundance in Soil Fungal Communities

Archaeorhizomycetes are also a major component of soil fungal communities from several studies. They were first detected as the novel fungal lineage Cluster 1 in alpine tundra soils (Schadt et al. 2003) at an average abundance of 15.2 % [19 out of 125 clones, see Supplementary material in Porter et al. (2008)]. In this study temporal dynamics of soil fungal communities were studied by sampling soils from below snow cover in winter and into the summer. Schadt and co-workers found that soil fungal community composition was stable from winter to spring but shifted significantly into summer, largely because of the dominance of Archaeorhizomycetes which made up 62 % of the clones sequenced from summer samples (Schadt et al. 2003). A cluster of Archaeorhizomycetes was identified accounting for 7 % of the winter samples, while none were identified in spring samples. Later Porter et al. (2008) examined four ecosystems: two different forests from within the Niwot Ridge Long-Term Ecological Research Site in Colorado (a tree line forest dominated by *Picea engelmannii* and *Pseudotsuga menziesii* and a montane forest with *Pinus contorta*, *Abies lasiocarpa,* and *Picea engelmannii*), one Costa Rican tropical forest on highly weathered P limited soil, and a temperate coniferous forest dominated by *Tsuga canadensis* in eastern Canada. They identified Archaeorhizomycetes, then Soil Clone Group 1, in 6.9–27 % of LSU clones derived from total soil DNA extracts from these ecosystems. Nevertheless, the highest abundance of Archaeorhizomycetes, 73–95 % of clones derived from soil DNA extracts, was detected by Castro et al. (2010) in a study of climate change effects on soil microbial communities associated with a reconstructed old-field plant community. The highest abundance was recorded in the wet treatment, which received 25 mm rain per week, and the lowest in the dry treatment, which received 2 mm per week. Apart from its exceptionally high abundance of Archaeorhizomycetes clones, the study by Castro et al. (2010) stands out in that sampling occurred late in the season (October 2006) which is close to the end of the growing season for Oak Ridge, TN. In addition to previously documented seasonal dynamics associated with plant

growth (Schadt et al. 2003), temporal dynamics of aboveground senescence, decreased carbon allocation to roots, and associated mycorrhizal fungi as well as root decomposition may be major drivers of relative abundance of Archaeorhizomycetes in soil fungal communities.

Assuming that abundance in soil DNA clone libraries reflects actual species abundance in terms of biomass or activity in soil, these studies demonstrate that members of Archaeorhizomycetes are major components of many soil fungal communities. While the internal transcribed spacer region (ITS) is the designated gene to describe fungal communities (Schoch et al. 2012), it has to be noted that Archaeorhizomycetes abundance estimates from studies targeting the ITS region are obscured by two specific mismatches in the binding site of the widely used reverse primer ITS4 (White et al. 1990). This can lead to great underestimation of Archaeorhizomycetes in soils. The three studies discussed above (Schadt et al. 2003; Porter et al. 2008; Castro et al. 2010) targeted the LSU region for amplification and sequencing of fungi from environmental samples by using reverse primers in the LSU region, such as nLSU1221R (Schadt et al. 2003) and TW13 (White et al. 1990), which do not appear to be biased against Archaeorhizomycetes. Therefore, the detected abundance of Archaeorhizomycetes in these studies might reflect their true abundance better than in studies using ITS4.

14.2 Archaeorhizomycetes in Alaska

The Alaskan boreal forest ecosystem is the best-documented ecosystem with respect to soil fungal communities (Taylor et al. 2010). After removing all singletons, 1,578 OTUs were identified in a dataset of over 52,000 sequenced clones. Among the 30 most common OTUs, only five were non-mycorrhizal and two of these can now be identified as Archaeorhizomycetes (labeled *Candida tepae* because no closer relatives were described at the time) (Taylor et al. 2010). Both these OTUs were found predominantly in the black spruce (*Picea mariana*) habitat. In an earlier study by Taylor et al. (2007), a putative Archaeorhizomycetes sp. (labeled OTU 76) was detected as the most abundant OTU, comprising approximately 25 % of the clones from a pooled DNA extract from humic black spruce soil. Two other OTUs (73 and 78) later identified as belonging to Archaeorhizomycetes were also identified in the study (Taylor et al. 2007). In Alaska, sequences belonging to Archaeorhizomycetes have been identified in ten studies targeting the ITS and LSU region (Table 14.1) as well as in two studies targeting the SSU region (Allison et al. 2008; Allison and Treseder 2008).

Table 14.1 Ten studies identify ITS and LSU sequences belonging to the Archaeorhizomycetes in Alaska

Study	Site	Rel. ab. (%)	# A-OTU	Tot# seq (OTUs)
Allison et al. (2010a)	Delta Junction	1	2	433 (113)
Allison et al. (2010b)	Delta Junction	2	6	327 (110)
Bent et al. (2011)	Bonanza Creek LTER	1[a]	2	152 (71)
Deslippe et al. (2012)	Toolik Lake	1	5	2,293 (777)
McGuire et al. (unpub.)	Delta Junction	13	7	156 (UK)
Taylor et al. (2007)	Bonanza Creek LTER	12	6	588 (148)
Taylor et al. (2008)	Fairbanks	8	6	456 (117)
TK	12 sites[b]	11	31	28,903 (2,537)
UP	9 sites	29	24	23,103 (3,093)
NAAT[c]	7 sites[b]	1	5	7,834 (1,834)

Sites from Interior and northern Alaska are represented. Relative abundance (Rel. ab.) is given as % sequences belonging to Archaeorhizomycetes out of all sequences from the study. Number of OTUs (#OTUs) calculated as described above. The total number of sequences available from each study is given under #sequences with total

[a]Bent et al. (2011) studied roots of spruce and birch. All the other sequences are obtained by cloning from total soil DNA extracts

[b]Archaeorhizomycetes sequences were detected at seven sites in the TKN study and in three sites in the UP study. See Fig. 14.2 for approximate locations of these sites

[c]In the NAAT study, sequence of Archaeorhizomycetes was detected at three sites along the North American Arctic Transect. Two of these are in Alaska (Fig. 14.2), and the third is on Prince Patrick Island in the Canadian High Arctic (Fig. 14.1)

14.2.1 Sequence and Statistical Analysis

One sequence representing each Archaeorhizomycetes OTU found in the ten studies (Table 14.1) was added to an alignment spanning the ITS and LSU rRNA followed by manual editing in Geneious Pro 5.5.5 (Biomatters Ltd.). Reference sequences of *Archaeorhizomyces finlayi* and Archaeorhizomycetes sp. FG15P2b were included in the alignment and AFTOL reference sequences for *Schizosaccharomyces pombe*, *Protomyces inouyei*, *Taphrina wiesneri*, *Taphrina deformans*, and *Saitoella complicate* were included as an out-group in the analysis. A maximum likelihood tree was derived from the alignment using RAxML-HPC2 on XSEDE, Cipres (Miller et al. 2010). Bootstrap support values were calculated from 1,000 iterations. Branches were collapsed to represent OTUs clustered in CAP3 at a 97 % similarity across the ITS. Most OTUs represented distinct and well-supported clades in the tree (Fig. 14.2). The exceptions include five cases where additional sequences were clustered within the OTU; these are indicated by + after the OTU number in the tree and one case when two OTUs could not be separated in the tree labeled OTU 7 and 8. Sequences from Allison et al. (2010a, b) comprised the LSU region only and were assigned to OTUs based on clustering within the tree. OTU5+, OTU11+, OTU12+, and OTU18+ all include one or two additional sequences with close to 97 % similarity across the ITS region to the other sequences in the OTU. In these cases sequence dissimilarity is mostly due to ambiguous base calls, i.e., N.

Fig. 14.2 Phylogeny of Archaeorhizomycetes in Alaska illustrated in a maximum likelihood tree derived from an ITS-LSU alignment. Reference sequences of *A. finlayi* and Archaeorhizomycetes sp. FG15P2b were included in the alignment and AFTOL reference sequences for *Schizosaccharomyces pombe, Protomyces inouyei, Taphrina wiesneri*, *Taphrina deformans,* and *Saitoella complicate* were included as an out-group in the analysis. Boot strap support values above 50 are shown in the figure. Putative species containing sequences from the Arctic NAAT study are indicated with *asterisk*. Five well-supported clades containing more than two putative species (I–V) are identified in the tree. Lineage-specific associations at the clade level with ecosystems are indicated with *asterisk* at the highest node, *orange* for mixed upland, and *green* for *black spruce*, and clades associated with soil horizons are indicated with a *filled box* at the highest node, *brown* for organic soil, and *gray* for mineral soil. Within clades significant lineages are shaded, and putative species with significant associations to soil horizon are marked with a *colored box* following the branch name. Indicator species are marked in the tree, for upland (I-Up), *black spruce* (I-BS), mineral soil (I-M), and organic soil (I-O)

Archaeorhizomycetes relative abundances, both as fraction of total sequences and as fraction of total OTUs, were derived from the Alaskan studies (Table 14.1) and analyzed by regression against latitude. Three studies were excluded from this analysis because they used the reverse primer ITS4 that has two mismatches for Archaeorhizomycetes, which may have resulted in an underestimation of abundance in these studies (Bent et al. 2011; Deslippe et al. 2012) and the unpublished study by McGuire and co-workers since we did not have data on total numbers of identified OTUs for this study.

To investigate species-environment relationships for Archaeorhizomycetes in Alaska, we carried out a variety of statistical analyses in PC-ORD 5 (McCune and Mefford 2006) with a combined species X site matrix from the TKN, UP, and NAAT datasets, utilizing abundance-based Bray-Curtis distances after applying a "general relativization" as recommended by McCune et al. (2002) when sample sizes differ among sites. In our first analysis, we tested whether communities differ according to major categorical habitat variables using multiple response permutation procedures (MRPP). We performed ordination of the sites using nonmetric multidimensional scaling (NMS). After 50 randomizations, a 3-dimensional solution was selected by PC-ORD using automatic mode. The best solution had a final stress of 18.86459 and a final instability of 0.00002 after 500 iterations. The relatively high stress indicates that the relationships among the sites were fairly weak, in agreement with the relatively small effect sizes (A) in the MRPP analysis (Table 14.2). Lastly, we carried out indicator species analyses to ascertain whether particular Archaeorhizomycetes taxa have a statistically significant preference for particular site categories. We analyzed only habitat, soil pH, and soil horizon, since those factors had the strongest correlations in the MRPP analyses. Furthermore, the phylogenetic distance of the Archaeorhizomycetes communities associated with (1) soil horizons O and E and (2) with habitats blacks spruce and mixed upland was analyzed separately using UniFrac (Lozupone et al. 2006). Samples from tundra, i.e., the NAAT study, were excluded from the UniFrac analysis because they represented only 20 sequences. Environments were clustered by jackknife analysis with 100 permutations, and lineage-specific analyses were performed to identify lineages with significant affiliation for certain environments. The analyses were performed using sequence abundance data excluding lineages with less than ten descendants and using presence–absences counts excluding lineages with less than four descendants. Using presence–absence data provides a more conservative measurement than abundance data that can be strongly driven by the more abundant species.

14.2.2 Diversity and Distribution of Archaeorhizomycetes in Alaska

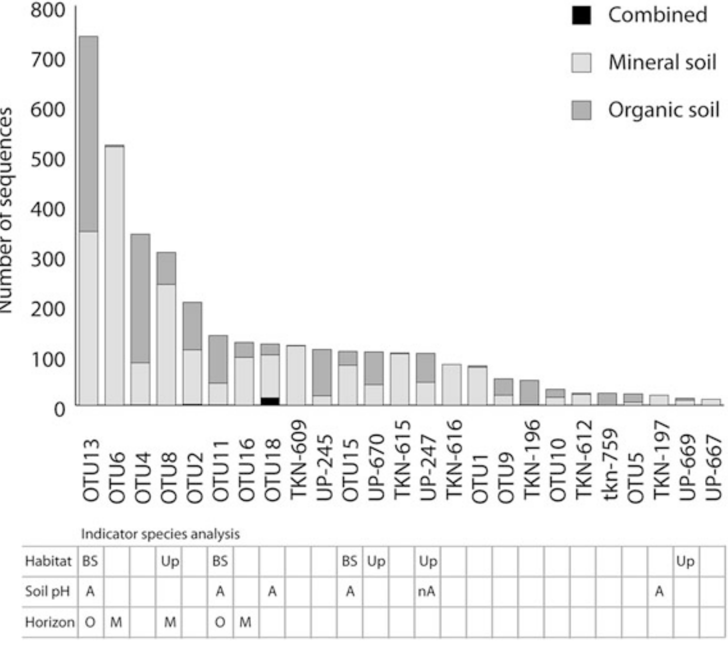
Earlier global estimates of Archaeorhizomycetes diversity encompassed ten putative species from Alaska (Rosling et al. 2011). In contrast, the current expanded phylogenetic analysis provides an estimate of 53 putative species of

Table 14.2 Multiple response permutation procedure results

Factor	Chance corrected within-group agreement, *A*	Probability of a smaller or equal delta, *p*
Habitat	0.09521448	0.00000000
Mineral soil pH	0.03604479	0.00000002
Soil horizon	0.03910208	0.00000041
Site moisture	0.03111664	0.00001152
Successional stage	0.02859186	0.00001808
Biome	0.01363238	0.00306595

Archaeorhizomycetes in Alaska. Eighteen of these putative species were detected in more than one study. Forty-six of these putative species were identified among 3,666 Archaeorhizomycetes sequences from the TKN, NAAT, and UP studies. Putative species identified as OTUs, i.e., in more than one study, as well as those represented by more than ten sequences, are named in the tree (Fig. 14.2). A rank abundance curve of the 25 most common Archaeorhizomycetes OTUs detected among the three studies (TKN, UP, and NAAT) demonstrates a classical pattern with a few very common species and numerous rare species (Fig. 14.3). The OTU TKN-615 is among species of average abundance (Fig. 14.3) and was identified as the type species *A. finlayi* (Fig. 14.2). This expands the known distribution of *A. finlayi* beyond its previous identification from Finland, Sweden, and New Hampshire (Rosling et al. 2011). Five well-supported major clades with more than two putative species (I–V) are identified in the tree (Fig. 14.2). The reference sequence of Archaeorhizomycetes sp. FG15P2b did not cluster with any Alaska sequence, supporting earlier indications that this species might be geographically limited to Europe (Rosling et al. 2011).

Across the ten field studies, relative abundance of the Archaeorhizomycetes ranged from 1 % to 29 % of the total fungal community identified by environmental sequencing (Table 14.1). Putative species in the Archaeorhizomycetes were detected from 18 sites in Interior and northern Alaska (Fig. 14.4). In Interior Alaska 9–25 OTUs per site were identified in the TKN study and 7–16 OTUs in the UP study. For the other published studies conducted in Interior Alaska, two to seven OTUs were identified per site (Table 14.1, Fig. 14.4). In the two studies conducted in northern Alaska, two and five OTUs were identified per site. Sampling at Prince Patrick Island in the Canadian Arctic yielded one OTU. Abundance and diversity of Archaeorhizomycetes was found to decrease from boreal to arctic ecosystems as demonstrated by a significant ($P < 0.002$) exponential decrease in relative number of OTUs and in relative abundance with increasing latitude. It is interesting to note that the single OTU from the Canadian Archipelago was identified as OTU4, which is the third most common putative species in Alaska (Fig. 14.3). This putative species also included sequences from the TKN study, a sequence representing the most common OTU (76) detected in Taylor et al. (2007) as well as sequences from Deslippe et al. (2012) and Taylor et al. (2008). Among all the putative



	OTU13	OTU6	OTU4	OTU8	OTU2	OTU11	OTU16	OTU18	TKN-609	UP-245	OTU15	UP-670	TKN-615	UP-247	TKN-616	OTU1	OTU9	TKN-196	OTU10	TKN-612	tkn-759	OTU5	TKN-197	UP-669	UP-667
Habitat	BS			Up		BS					BS	Up		Up										Up	
Soil pH	A					A		A			A			nA									A		
Horizon	O	M		M		O	M																		

Fig. 14.3 Rank abundance of putative Archaeorhizomycetes species in Alaska, measured as number of sequences assigned to each OTU from the three studies NAAT, UP, and TKN. Sequence origin with respect to soil horizon is illustrated in *dark gray* for organic soil, *light grey* for mineral soil, and *black* for combined samples. Below each OTU results from the indicator species analysis are listed for habitats [*black spruce* (BS) or mixed upland (Up)], soil pH [acid (A) or nonacid (nA)], and soil horizon [organic (O) or mineral (M)]. TKN-207 is identified as an indicator species for acidic soil but does not appear in the figure because it is only represented by ten sequences among all studies

Archaeorhizomycetes species identified here, only one common generalist (OTU4) is detected in among the arctic samples analyzed.

14.2.3 Habitat Specificity

Using MRPP, we found that the composition of the Archaeorhizomycetes community was correlated with several habitat-related environmental variables (Table 14.2). The strongest correlation was with our variable "habitat," by which we distinguished black spruce boreal forest, upland boreal forest, and tundra. We also found highly significant correlations with pH of the mineral soil at these sites (acidic, nonacidic), as well as site moisture (wet, mesic, dry), successional stage (early, middle, late), and biome (boreal forest vs. arctic). These patterns are in accordance with previous observations from these sites where total fungal

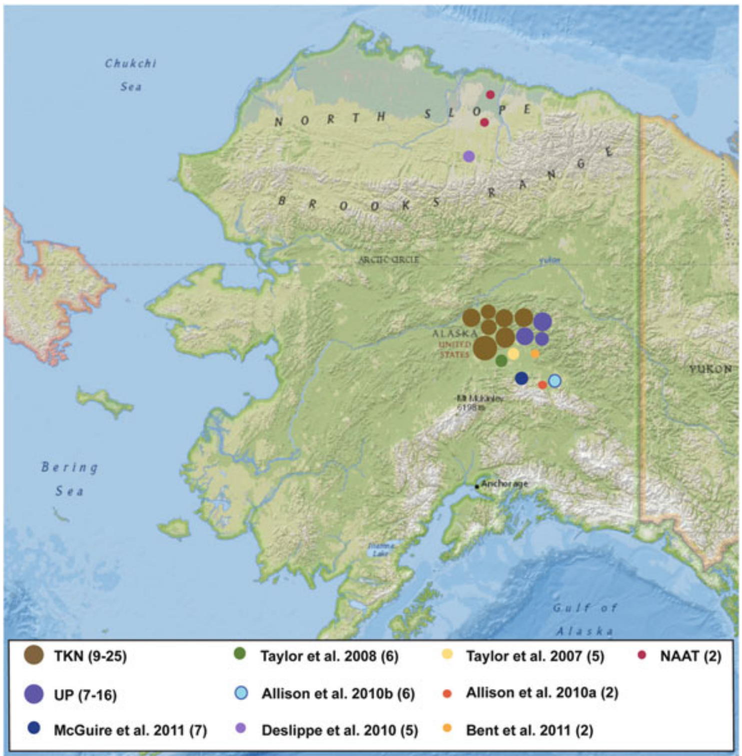


Fig. 14.4 Schematic overview of study sites for ten studies that have identified sequences belonging to the Archaeorhizomycetes in Alaska. Size of *dots* illustrates the number of putative Archaeorhizomycetes species, i.e., OTUs defined at 97 % sequence similarity across the ITS region. Numbers of species per study are also given as values in parenthesis after the study name in the legend

communities were also found to be different among these habitats as well as between the two soil horizons (Taylor et al. 2010). However, it should be noted that these categories are confounded in the dataset. For example, most of the acidic sites are also black spruce forest, and the only early successional sites were in upland boreal forest. Thus, it is most parsimonious to consider habitat (forest type) as the overriding factor, with a set of intercorrelated environmental factors that are associated with habitat. However, because horizon was analyzed independently in both black spruce and upland datasets, the significant correlation with this factor can be interpreted more directly: the composition of the Archaeorhizomycetes community differed between the organic and mineral horizons. In concordance with the MRPP analyses, NMS demonstrated that the environmental factor that best coincided with fungal community variation across the sites was habitat (Fig. 14.5).

The indicator species analyses (Table 14.3, Figs. 14.2, and 14.3) suggest that 12 taxa are specialists. For example, TKN204 in OTU13 is an indicator of acidic

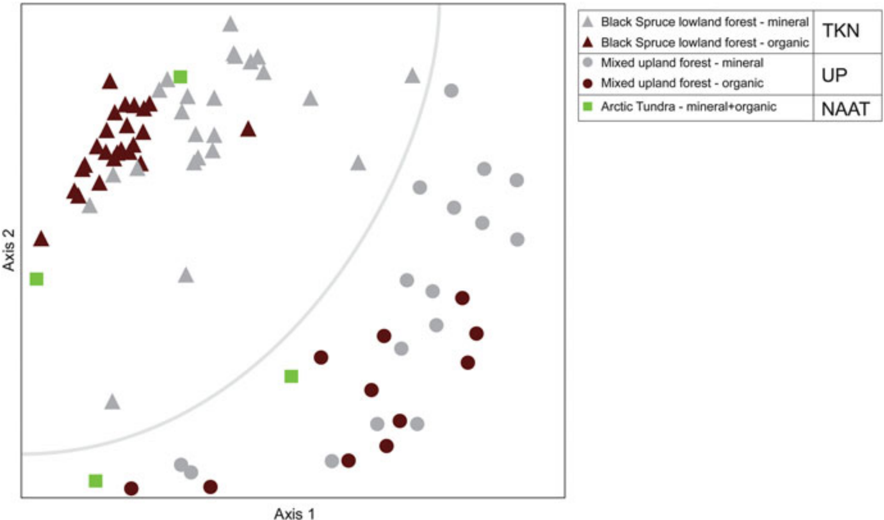


Fig. 14.5 NMS ordination of Archaeorhizomycetes communities from three studies in Alaska (TKN, UP, and NAAT). *Triangles* indicate *black spruce* forest sites, *circles* indicate upland boreal forest sites, and *squares* indicate tundra sites. *Brown symbols* specify organic horizon, and *gray symbols* designate mineral horizons. Notice the strong grouping of *black spruce* vs. upland boreal forest sites

soil in black spruce forests and prefers the organic horizon. In contrast, UP247 is an indicator of nonacidic soils and upland forests, but did not reveal a horizon preference. As with the MRPP analyses, it should be noted that many of these environmental factors covary across sites, meaning that it is impossible to infer which factor drives the observed correlation. For example, it may be that UP247 has a preference for particular tree species and that the apparent preference for non-acidic soils is simply due to the fact that most of the nonacidic sites are also upland forest, where these tree species occur.

Jackknife analysis in UniFrac demonstrates that the environment soil horizons O vs. E as well as ecosystems black spruce vs. mixed upland harbor phylogenetically distinct communities of Archaeorhizomycetes (100 % node support for both presence–absence and sequence abundance data). Lineage specificity towards ecosystems, which were based on presences–absences, is highlighted in green for black spruce and orange for mixed upland (Fig. 14.2). Highlighted clusters are not only significantly associated ($P < 0.01$) but exclusively associated with their ecosystem. Taking sequence abundance into account, significant lineages are identified at higher order as illustrated by colored asterisks at the highest significant node (Fig. 14.2). Based on the latter, Clade III and IV are significantly associated with the black spruce ecosystem, while Clade V and a clade with the two putative species OTU 9 and TKN-612 are significantly associated with the mixed upland ecosystem. Ecosystem specificity among lineages is a strong driver for the observed differences in Archaeorhizomycetes

Table 14.3 Results of indicator species analysis for the three categories: habitat, soil pH, and soil horizon

Taxon	Observed indicator value (IV)	Mean	Standard deviation	p	Factor	Preference
OTU 13 TKN204	72.9	23.9	7.45	0.0002	Habitat	Black spruce
UP247	55.2	15.2	6.85	0.0008	Habitat	Upland
OTU 11 TKN198	47.9	18.4	7.36	0.0064	Habitat	Black spruce
OTU 7 and 8 TKN206-UP242-UP244	37.7	20.9	7.67	0.0378	Habitat	Upland
OTU 15 TKN202	31.2	14.6	7.17	0.0396	Habitat	Black spruce
UP669	20.7	8.7	5.21	0.0402	Habitat	Upland
UP670	24.1	9.7	5.78	0.0446	Habitat	Upland
TKN203	41.6	16.5	4.67	0.0002	Soil pH	Acidic
OTU13 TKN204	52.9	26.6	4.53	0.0006	Soil pH	Acidic
OTU15 TKN202	33.7	13.7	3.89	0.0008	Soil pH	Acidic
UP247	28.6	14.4	4.09	0.007	Soil pH	Nonacidic
OTU 11 TKN198	31.8	18.9	4.17	0.0116	Soil pH	Acidic
TKN207	11.1	4.5	1.94	0.029	Soil pH	Acidic
TKN197	11.1	4.4	1.99	0.0332	Soil pH	Acidic
TKN205-UP979	37.5	16.2	7.37	0.0188	Soil horizon	Mineral
TKN204	44.5	23.8	7.39	0.022	Soil horizon	Organic
TKN198	37.6	18.3	7.34	0.0278	Soil horizon	Organic
TKN206-2	38.9	20.8	7.81	0.0352	Soil horizon	Mineral
TKN200-U	32.4	16.7	7.18	0.0442	Soil horizon	Mineral

community composition that we observe in the Alaska dataset. This pattern is most likely driven by host specificity as previously demonstrated for several putative species in the class (Rosling et al. 2011). OTU13 is significantly associated with the black spruce ecosystem (Fig. 14.2), and this putative species shared reference sequences with the previously listed OTU3 specifically associated with spruce root (Rosling et al. 2011). The same is true for OTU2 that was previously listed as the spruce-specific OTU13 (Rosling et al. 2011). The tundra habitat is only represented by four putative species, OTU2, 4, 15, and 18+, which are all associated with the black spruce habitat.

For the environmental variable soil horizon, lineage-specific analysis, based on presence–absence, identified Clade V as significantly associated with mineral soil ($P = 0.0094$) and Clade I as significantly associated with organic soil ($P = 0.0064$). However, the association with soil horizon is not exclusive to the same extent as the association with ecosystems discussed above. Taxa and lineages with significant associations with organic horizons are also identified in mineral soils, while at least eight putative species appear to be restricted to mineral horizons (Fig. 14.3). Thus, sequence abundance data, as opposed

to presence–absence, provide a more informative image of lineage significance towards soil horizon. Significant lineages are highlighted in gray for mineral soil and brown for organic soil (Fig. 14.2). Putative species with significant associated with a soil horizon that is found within a clade without significant associated to the same horizon are indicated by a colored box following the taxa name (Fig. 14.2). Three identified indicator species for mineral soil (I-M), OTU6, OTU8, and OTU16, were all found in lineages with a significant association with mineral soil (Fig. 14.2). The same is true for OTU 11+ and OTU13 which were identified as indicator species for organic soil (I-O) and found in lineages with significant association with organic soil.

Clade IV was significantly associated with the black spruce ecosystem when considering sequence abundance data but not when using the presence counts. The two sequences in this clade were derived from the mixed upland ecosystem, dominated by white spruce (*Picea glauca*), indicating a strong association with spruce for this clade. Clade IV is split into two well-supported clades with contrasting soil horizon preferences. OTU13 is the most abundant taxon in the current dataset. It is an indicator species for organic soil in the black spruce ecosystem, and the lineage is significantly associated with organic soil (Fig. 14.2). Yet OTU13 is still frequently detected in mineral soil (Fig. 14.3). The sister clade which encompasses *A. finlayi*, on the other hand, is significantly associated with mineral soil, and 94 % of all sequences in this clade were derived from mineral soil. This observation is supported by the initial isolation of the *A. finlayi*-type culture from a coniferous root collected in mineral soil at the interface between illuvial and eluvial soil horizons in a podzol soil profile (Rosling et al. 2003).

Clade I was significantly associated with organic soil. Within this clade, contrasting soil horizon specificity was detected among sister species, i.e., TKN-197 and TKN-196 associated with organic soil vs. TKN-759 associated with mineral soil (Fig. 14.2). Similar to patterns of vertical partitioning observed for sister species of Rhizopogon (Beiler et al. 2012), patterns of differential distribution between soil horizon may well be the result of ongoing substrate competition among closely related species.

Overall, the relative abundance of Archaeorhizomycetes was higher in mineral soils, close to 9 % of all clones, compared to roughly 6 % in organic soil across the three studies from Alaska discussed here. Higher relative abundance in mineral soil may reflect lower abundance of other taxa rather than an absolute increase in Archaeorhizomycetes in mineral soil. However, lineage specificity towards mineral soil was common within clades II, IV, and V, suggesting that a large proportion of the diverse class is well adapted to conditions in mineral soil. This observation is supported by previous findings from Lindahl et al. (2007) where fine separation of horizons from a boreal forest floor followed by fungal community characterization using T-RFLP identified six putative Archaeorhizomycetes (then Ascomycete group G) occuring in all soil layers except for the layer of new litter. Putative Archaeorhizomycetes species had different but overlapping patterns of occurrence with most taxa being identified in the upper humus layer. Two putative species,

C2y_8.2 (JN032481) and A2z_5.11 (JN032482) became increasingly common in mineral soil horizons. The latter formed part of OTU9 (Rosling et al. 2011), which represents the putative species OTU18+ in the current analysis.

14.3 Life Strategies in Archaeorhizomycetes

A shift in community composition towards dominance by Archaeorhizomycetes in summer samples was interpreted as an indication that the class depends on carbon derived from root exudation (Schadt et al. 2003). Furthermore, the two cultured representatives of Archaeorhizomycetes, *A. finlayi* and Archaeorhizomycetes sp. FG15P2b, were both isolated from surface-sterilized root tips, further suggesting that species in the class are intimately associated with roots. There are, however, indications that the class is not dependent on living roots for their carbon supply. Five and 14 days after severing of roots in a pine forest, the relative abundance of Archaeorhizomycetes (then Clone Group 1) remained close to that in the control samples with an average abundance of 14 % of total fluorescence as quantified by T-RFLP (Lindahl et al. 2010). That study analyzed abundance in soil DNA extracts, and targeting the active community might give a different representation of Archaeorhizomycetes. Furthermore, the study by Castro et al. (2010) stands out with its exceptionally high relative abundance of Archaeorhizomycetes clones (up to 95 %) of the soil fungal community at the end of the growing season. Possible temporal dynamics of Archaeorhizomycetes associated with aboveground senescence decreased carbon allocation to roots, and associated mycorrhizal fungi as well as root decomposition may be well worth studying in the future.

Species in Archaeorhizomycetes have the ability to grow inside roots as well as on pure carbon sources of varying complexity (Rosling et al. 2011). Sequences of Archaeorhizomycetes have also been detected in decaying wood. Rajala et al. (2011) studied the active fungal community of decaying spruce logs using a combination of DGGE and sequencing from environmental rDNA and rRNA extracts. A sequence representing Archaeorhizomycetes was obtained from strongly decayed spruce logs. These observations in combination with the shear abundance of Archaeorhizomycetes in many soils suggest that these fungi play an important role in the cycling of carbon derived from living or dead roots in soil. Neither mycorrhizal nor pathogenic interactions have yet been documented for the type species *A. finlayi*. Whether Archaeorhizomycetes are directly associated with roots along a trophic continuum from symbiotic–endophytic–saphrotrophic–pathogenic interactions or are secondarily associated through interactions with other root-associated fungi remains unknown. Taking into account that Archaeorhizomycetes is an ancient class of fungi, there is good reason to acknowledge that different life strategies may be represented among species in the class and that no single ecological role may be assigned to the class.

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Chapter 15
Methods in Fungal Genetics

Kevin McCluskey and Aric Wiest

15.1 Introduction

15.1.1 Evolution of Modern Gene Manipulations

Whether in saving starter for sourdough bread or making koji mold for Sake fermentation, humans have been manipulating fungi for their own purposes for millennia. The Tyrolean iceman, Ötzi, had fungi among his personal items although whether for medicinal or practical use is still debated (Rollo et al. 1995). With the development of modern germ theory and the exposition of Koch's postulates, practical microbiology included saving and characterizing pure cultures of microorganisms. Even before Fleming discovered his *Penicillium* strain (Bennett and Chung 2001) that led to modern pharmaceutics, culinary strains were selected for high spore production, lack of toxic metabolites, flavor profiles, purity, and stability (Machida et al. 2008). It can be argued that once strain improvement for higher antibiotic production began, modern biotechnological manipulation was on a natural continuum, and while the specific techniques have changed, the general aims remain the same. These aims include high productivity, lack of contaminating side products, purity, and stability.

15.1.2 Research Versus Commercial Applications

As biological sciences went from being purely descriptive, and experimental biology became practical, fungi were brought into the laboratory for characterization and as model systems for larger biological inquiry. Among the foundational

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6_15,
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discoveries made using model fungal organisms are the one gene-one enzyme hypothesis (Beadle and Tatum 1941), the existence of suppressor mutations (Tatum and Perkins 1950), multifunctional gene clusters, and regulatory mutations (Horowitz et al. 1961). In recent years, fungi continue to be valuable models for population genetics, genome organization, epigenetic gene modification, circadian rhythms, and host-pathogen interactions.

Commercial use of characterized and improved fungal material touches on many aspects of agriculture, medicine, food and fiber processing, and increasingly in the day-to-day activities of a modern lifestyle (Bohlin et al. 2010). Whether it be biocontrol of insect pests or invasive non-crop plants, the manipulation of field populations of toxin-producing or pathogenic organisms, the production of industrial chemicals, bio-processing, or the production of enzymes or pharmaceuticals, genetically characterized and manipulated strains represent the foundation and reference against which all progress is measured (OECD 2001).

Many of the research uses of fungi are removed from practical applications, although both approaches increasingly rely on the use of genetically manipulated strains. It is important in this context to emphasize that genetically manipulated strains may be generated by meiotic genetics, mitotic recombination, and by DNA-based genetic transformation using genetic constructs produced in vitro.

15.1.3 GMO Organisms and Regulatory Status

Materials collected in the environment, whether from intact wilderness, from agricultural, or even urban settings, are considered to be genetically wild type, and strains that have been derived from them by classical mutagenesis or by Mendelian or even mitotic recombination are evaluated and regulated according to the same criteria. Materials that have been generated by genetic manipulation using engineered or synthetic molecules are subject to regulation as hazardous biological materials. Moreover, shipment of GMO materials is governed by the same guidelines as dangerous goods. At the time of the writing of this review, this circumstance is in a state of flu15. The Cartagena Protocol on Biosafety to the Convention on Biological Diversity (http://bch.cbd.int/protocol/) is an international treaty negotiation with purview over the safe handling, transport, and use of GMO organisms, which they describe as “living modified organisms.” This protocol further describes its purpose as the protection of biodiversity from the potential risks of living modified organisms. It entered into force in September 2003, having been ratified by 50 countries. Regardless of the purpose, this protocol will have implications for the use of modified organisms in research, agriculture, and in the areas of pharmaceutical and industrial research and production.

15.1.4 Scope

The present chapter will describe the development of tools for manipulation of filamentous fungi with emphasis on tools for research application. It will describe the evolution of modern molecular genetic tools and include circumspection on their characteristics. Emphasis will be placed on the use of materials in the collection of the Fungal Genetics Stock Center, and description of these materials will be elaborated, to the extent that it provides insight into directions of research over many years.

15.2 Classical Genetic Manipulation

Once fungi had been domesticated and put to practical uses, and simultaneous with the rediscovery and validation of Mendel's work on pea genetics by de Vries and others in the late nineteenth century (Hurst 2009), fungi were subject to investigations of their genetics both to explore the nature of genes and as a practical means of strain improvement. While some organisms were studied just to identify principles of genetics (Shear and Dodge 1927; Blakeslee 1904), others were studied primarily because of their practical application or impact (Takamine 1914; deBary 1853).

15.2.1 Mendelian Genetics

The development of model systems for filamentous fungi was based on the ability to conduct Mendelian genetics. The demonstration of segregation of mating type was made in a number of fungal systems including *Neurospora* (Lindegren 1932), *Schizophyllum*, *Ustilago*, *Sordaria*, and yeast (Kronstad and Staben 1997). Similarly, mating type was characterized in a number of water molds, some of which are no longer considered fungi. These included *Allomyces* (Emerson and Wilson 1949), *Achlya* (Raper 1939), and *Phytophthora* (Judelson et al. 1995). In parallel, mating and other characteristics were described for slime molds, and these had significant parallels to fungal systems (Bonner 1944). Classical genetics has been foundational for the understanding of fungal biology and for many years was the best way to characterize interesting traits. This also included extensive study of the mechanisms of genetics, the nature of intra- and intergenic recombination, as well as population and quantitative genetics. In the most well-characterized systems, such as *Neurospora*, *Aspergillus*, yeast, and numerous plant-pathogenic fungi, genetic maps were constructed demonstrating the number of linkage groups and the relationship of markers on these linkage groups. This was corroborated by cytogenetic analysis of stained chromosomes, (McClintock 1945) although for many fungi, the number

of chromosomes is underestimated because most of the chromosomes are too small for classical microscopic analysis as demonstrated by electrophoretic karyotype analysis (Mills and McCluskey 1990). For example, *Neurospora* has seven linkage groups and also seven microscopically visible chromosomes, while *Ustilago* has as many as 20 electrophoretically resolvable chromosomes (Kinscherf and Leong 1988) but only 4 that are visible in cytological preparations (Lindegren 1948). Moreover, while many years of mutagenesis and characterization have yielded as many as 1,200 markers on the *Neurospora* genetic map (Galagan et al. 2003), whole-genome sequence has identified closer to 10,000 genes in this fungus. The systematic gene deletion program for *N. crassa* (Colot et al. 2006) has produced gene deletion mutants for over 8,000 unique ORFs, and of these, 1,500 are only available as heterodikaryons, suggesting that these ORFs are essential. This suggests that the saturation mutagenesis of many years was successful in mutating most ORFs for which a phenotype could be recovered. Moreover, these KO strains effectively carry an antibiotic resistance marker at every locus, and this can be used for fine structure mapping (Hammond et al. 2012).

15.2.2 Non-Mendelian Genetics

Because the demonstration of Mendelian genetics requires appropriate mating pairs or specific conditions to produce a teleomorph, some fungi were considered to be asexual, although mating-type alleles and even sexual reproduction have been identified for some species (Arie et al. 1997; O'Gorman et al. 2008). For these, the ability to carry out mitotic recombination was a valuable tool for pseudo-genetic manipulation. For example, the first markers mapped in *Aspergillus nidulans* were characterized by mitotic mapping (Pontecorvo 1956). Other approaches to non-Mendelian genetics included protoplast fusion between noncompatible strains and species. This leads to the production of potentially aneuploid strains, and the reduction of this aneuploidy can generate stable strains with traits not available through natural processes (Peberdy 1979). In a similar vein, interspecies crosses can be carried out for some organisms. The spore-killer element from *Neurospora intermedia* was introgressed into *N. crassa* (Turner and Perkins 1979) to allow better characterization. This element is carried on a 1.9 Mb region on the left arm of contig 3 presumably including the entire meiotic drive element along with other sequences (McCluskey et al. 2011).

Similarly, the characterization of strains carrying aneuploid or rearranged chromosomes has been useful both with regard to the insight it provides into fungal chromosome biology and also as a tool for characterization of gene location and epigenetics (Perkins 1997). Supernumerary chromosomes, sometimes referred to as B chromosomes (Hurst and Werren 2001) or, more recently, lineage-specific chromosomes (Ma et al. 2010), have been characterized in a number of fungi (Covert 1998). These chromosomes do not follow typical Mendelian genetics and are historically considered to be segregation distorters (Jones et al. 2008). The

distribution of these elements is not uniform, and while some fungi, such as *Neurospora*, do not tolerate foreign DNA (Windhofer et al. 2000), others, such as *Nectria haematococca* (Coleman et al. 2009) or *Fusarium oxysporum* (Ma et al. 2010), have a significant portion of their genomes on these dispensable chromosomes (Croll and McDonald 2012).

Now characterized in many organisms, epigenetic phenomena were characterized in filamentous fungi beginning with the demonstration of the mutagenic phenomenon induced by duplicated genes and known as RIP (Cambareri et al. 1989). Similarly, vegetative silencing in fungi was demonstrated by silencing of genes for carotenoid biosynthesis (Cogoni et al. 1996). Epigenetic phenomena are now known to be present in myriad fungi (Selker 1997).

15.2.3 Mutagenesis

Early research with fungi leading to such hallmark discoveries as the one gene–one enzyme hypothesis and intragenic recombination was carried out with filamentous fungi. Among these were the demonstration of spore color mutants in the homothallic species *Sordaria* (Sang and Whitehouse 1983), light sensing in *Phycomyces* (Delbruck and Meissner 1968), and auxotrophy and morphological anomalies in *Neurospora* (Beadle and Tatum 1941). For many fungi, mating type was the most reliable character subject to Mendelian analysis in fungi, and this one area was disturbingly not amenable to mutational analysis.

In *Neurospora*, and other fungi, filtration enrichment was developed as a technique to increase the likelihood of identifying appropriate mutant strains (Woodward et al. 1954). In this ingenious approach, heavily mutagenized spores are allowed to germinate in liquid culture and then subject to multiple cycles of filtration over loosely woven cotton cloth or other coarse matrix. Conidia that germinated either in minimal medium, or in special cases under selective pressure such as elevated temperature, were trapped in the filter, and conidia with a nutritional or other requirement passed through the filter. Subsequent culture of these defective conidia in complete medium or at reduced selection pressure identified mutants with a frequency that is much higher than simple mutagenesis.

Because of the frequency of inducing secondary mutations, it has always been best practiced to backcross mutants to a well-known wild type. Estimates on the number of crosses suggest that as many as ten backcrosses may be necessary to purify a unique mutation (Leslie 1981), although in practice this large number of backcrosses was associated with a 20-map unit recombination block around mating type.

Whole-genome analysis of classical mutant strains of *N. crassa* has shown the impact of backcrossing into the reference genome (Table 15.1). Where the number of backcrosses was known for classical mutant strains, the lowest divergence from the reference genotype was seen in strains that had been backcrossed multiple times into the reference genotype. This analysis rests on the fact that most SNPs were not

Table 15.1 Impact of backcrossing on frequency of polymorphisms detected in whole genome sequence from classical mutant strains of *N. crassa*

Strain	Marker	Mutagen	Backcrosses	Total SNPs	% max SNPs	# nonsense mutations	Reference
106	*com*	UV	3	23,579	12.5	18	Perkins and Ishitani (1959)
305	*amyc*	–	3	90,195	47.9	67	Atwood and Mukai (1954)
309	*ti*	X-rays	3	13,274	7.0	11	Perkins (1959)
322	*ty-1*	Spontaneous	–	142,489	75.7	95	Horowitz et al. (1961)
821	*ts*	Spontaneous	–	188,346	100.0	122	Nakamura and Egashira (1961)
1211	*dot*	Spontaneous	3	20,493	10.9	19	Perkins et al. (1962)
1303	*fi*	Spontaneous	–	59,356	31.5	35	Perkins et al. (1962)
1363	*smco-1*	Mustard	–	146,641	77.9	137	Garnjobst and Tatum (1967)
2261	*do*	UV	3	44,839	23.8	37	Perkins et al. (1962)
3114	*Sk-2*	Introgression	1	41,085	21.8	31	Turner and Perkins (1979)
3246	*fs-n*	Spontaneous	–	21,533	11.4	14	Mylyk and Threlkeld (1974)
3562	*mb-1*	UV	–	106,533	56.6	78	Weijer and Vigfusson (1972)
3564	*mb-2*	UV	–	47,981	25.5	36	Weijer and Vigfusson (1972)
3566	*mb-3*	UV	–	37,516	19.9	27	Weijer and Vigfusson (1972)
3831	*ff-1*	Spontaneous	–	22,961	12.2	15	Tan and Ho (1970)
3921	*tng*	Spontaneous	2	80,311	42.6	84	Springer and Yanofsky (1989), Howe and Benson (1974)
7022	*fld*	Spontaneous	–	78,991	41.9	45	Perkins et al. (1962)
7035	*per-1*	UV	3	18,487	9.8	13	Howe and Benson (1974)

unique and the result of the particular mutagenesis. Rather, they were found in multiple strains and seem to be associated with the multiple lineages used in the *Neurospora* research community. Many of the same polymorphisms seen in these classical mutant strains were also found in an independent analysis of a separate classical mutant strain (Pomraning et al. 2011).

Interestingly, nonsense mutations were common in classic mutant strains. The distribution of nonsense mutations was correlated with the lineage of the strain and inversely related to the number of backcrosses. The strains with the fewest numbers of nonsense mutations had all been backcrossed three times, while the strains with the most polymorphisms had not been backcrossed into the reference genome lineage (Table 15.1), emphasizing the importance of multiple generations of backcrossing in strain construction.

For many years, strain improvement included mutagenesis. Penicillin-producing strains were a key example of the impact of strain improvement (Sermonti 1959). Useful yields of antibiotic increased by several orders of magnitude during classical strain improvement. Subsequently, whole-genome analysis of *A. niger* strains subject to classical strain improvement showed the impact of strain improvement on the genome (Andersen et al. 2011). This study revealed many differences between the improved and reference genome strains including a high level of nucleotide polymorphisms, significant genome rearrangements, deletions, and even sequence unrelated to the reference genome. Transcriptome analysis also indicated that significant changes in regulation of gene expression had occurred.

Numerous recent efforts have characterized classical mutant strains and among them are the studies of nine *Neurospora* strains carrying classical mutations in genes responsible for pigment biosynthesis. These strains were characterized by direct sequence analysis of the *albino-2* gene (Diaz-Sanchez et al. 2011). One of the strains produced an aberrant pigment while others were albino. The red pigment-producing strain had multiple non-synonymous mutations as well as other synonymous ones. Several of the mutants produced truncated or shortened polypeptides and these mutants were variously completely albino or leaky. Only some polymorphisms at *al-2* were shared among multiple strains suggesting that these mutant strains were not all generated in the identical genetic background, similarly to that which was seen among the classical mutant strains subject to whole-genome analysis. Similarly, well-mapped mutations at the *ad-8* locus in *Neurospora* were used to establish that intragenic recombination was responsible for some instances of allele complementation (Ishikawa 1962). A subset of these mutations were characterized by targeted sequence analysis (Wiest et al. 2012a), and unlike the mutants at *al-2*, the *ad-8* mutants did not contain multiple changes, nor did they show any influence of founder effects.

15.3 Transformation

Originally demonstrated in *Streptococcus* in response to fundamental questions of the nature of the material of inheritance (Alloway 1932), the ability to transfer traits by introducing foreign DNA, either natural or from synthetic origins, among strains is foundational to every modern research system. While the techniques for accomplishing this transformation vary, all rely on the ability to introduce foreign DNA into a strain and have that strain express that DNA as though it were part of the natural genome. For some organisms, this is relatively straightforward, and the introduced DNA can become part of the nuclear genome. For other organisms, gene silencing and even mutation can limit the ability to mobilize and express foreign genes at high levels. Also, and in a manner similar to the requirement for backcrossing strains arising from explicit mutagenesis, transformants often need to be purified. This is especially important for organisms where the transforming cells are multinucleate, and nuclei that are not transformed can persist in hyphae

that are shared with a nucleus expressing the selectable marker gene. Moreover, transformation itself is mutagenic, and this has been shown both for insertion-associated mutations (Perkins et al. 1993) and also for second-site mutations (Keller et al. 1990).

15.3.1 Protoplast/Polyethylene Glycol

Protoplast fusion, whether in the presence of synthetic DNA or between cells carrying unique traits, has been used for strain manipulation and improvement for many years (Turgeon et al. 2010). The best results are available when high-quality protoplasts are available (Daboussi et al. 1989). The distinction between spheroplasts and regenerable protoplasts lies in the fact that protoplasts contain all the requirements to form a new colony while spheroplasts are merely osmotically sensitive liquid-filled micelles (Peberdy 1987).

The production of protoplasts requires enzyme cocktails to remove the cell wall polysaccharides and to make the cell permeable to DNA in the presence of polyethylene glycol (PEG) or similar polymer (Peberdy 1987). While generic enzyme cocktails may work for some species, other species require custom enzymes or other pretreatment. *Neurospora*, *Saccharomyces*, *Ustilago maydis*, and *A. nidulans* are generally amenable to protoplasting with generic enzyme cocktails, although even these amenable species are vulnerable to inconsistencies in enzyme specificity (de Bekker et al. 2009). Some organisms, such as *U. hordei*, *A. fumigatus*, or *Schizophyllum*, require specific enzyme cocktails. While this is sometimes accomplished by the addition of specific purified enzymes, the best protoplasting cocktails are generated by growing the enzyme-producing fungus, typically *Trichoderma* or *Aspergillus niger*, on purified cell walls from the target organism (Peberdy 1987). For example, to produce cell wall-degrading enzymes to make protoplasts from *S. commune*, one would first grow a large batch of *S. commune* mycelia and then use the purified cell walls of the *S. commune* strain as substrate to culture the *Trichoderma* or *A. niger* strain from which the cell wall-degrading enzymes are to be extracted.

Another approach is to use a strain that either constitutively or conditionally fails to produce a cell wall. In *N. crassa*, a strain known as slime contains three genetic lesions that combine to produce a wall-less strain (Selitrennikoff 1979). Regardless of how the protoplasts are produced, all transformation techniques rely on the use of PEG. The role played by the PEG in transformation was analyzed and compared to other molecules, and it was considered that the PEG functioned in concentrating the DNA and that it does not directly cause protoplast fusion (Kuwano et al. 2008).

15.3.2 Electroporation

This technique takes advantage of the ability of a pulse of electrical energy to create pores in biological membranes which allows DNA to enter a cell (Dower et al. 1988). While the exact mechanism by which electroporation facilitates DNA uptake and entry into the nucleus is unknown, alternate theories include the passage of DNA through pores (Levine and Vernier 2010), the formation and uptake of vesicles (Kawai et al. 2010), or even the electrophoretic acceleration of DNA in an electric field.

While the characteristics of the electrical discharge are subject to discussion (Weaver 1995) and the ideal system for electroporation should have a square discharge wave, practical issues mean that a logarithmic discharge is more readily achieved and produces adequate transformation efficiencies (Chen et al. 2006).

15.3.3 Chemical

Typically involving lithium acetate and modeled after bacterial transformation (Chung et al. 1989), some species can be readily transformed by preparation of competent cells using salt treatment. This has been shown not only for yeast-like fungi including Saccharomyces but also in *Microbotryum violaceum* (as *U. violacea*) (Bej and Perlin 1989). The role of lithium, the preference for different lithium salts, and the ability of different cations to replace lithium have been exhaustively studied (Kawai et al. 2010).

15.3.4 Biolistic

In some cases, DNA can be directly introduced to cells using microscopic pellets coated with the DNA one wishes to introduce. Because of the complexity of this protocol and because of the relatively low yield, it is preferential to use another approach where available. In some cases, biolistic transformation is the only option, and in these cases, it can be extremely useful (Olmedo-Monfil et al. 2004).

15.3.5 Agrobacterium

Many species of fungi, even filamentous fungi, can be transformed by conjugation of special plasmids. This is accomplished by co-culturing the target organism with *A. tumefaciens* cells carrying a plasmid mobilized by the presence of so-called T-DNA regions (Betts et al. 2007). The DNA that is transferred into the fungal cell

integrates randomly into the genome and generates a tagged integrant where the presence of the T-DNA marks the insertion (de Groot et al. 1998). Strains transformed with this method require careful evaluation to assure the presence of a unique insertion. Moreover, the stability of the inserted DNA has not been evaluated in all target species. This may be especially relevant for species where meiotic gene silencing or mutation depends on the presence of repeated DNA. The ability to transfer DNA via Agrobacterium was exploited in the generation of tagged-integrant mutants of *Magnaporthe grisea*, and 48,000 such strains were generated and phenotyped (Tucker and Orbach 2007; Soderlund et al. 2006).

15.4 Selectable Markers

Transformation by foreign DNA is relatively inefficient (Fincham 1989) with 1–100 transformants per microgram of transforming DNA and per 10^7 recipient cells being not uncommon. Because of this, it is not practical to screen for cells that have foreign DNA, and rather selectable markers, which allow only the cells carrying and expressing foreign DNA to grow, are employed. These markers vary in their characteristics, but all allow the growth of only cells expressing the marker gene. In addition to a robust selectable marker, appropriate fungal promoters and terminators are required for stable transformation. For many markers, the *Aspergillus nidulans* TrpC promoter and terminator were used (Yelton et al. 1984).

15.4.1 Auxotrophic Complementation

The first demonstration of transformation by complementation of auxotrophy was made by culturing cells of a Neurospora strain that required inositol with DNA from an inositol prototrophic strain (Mishra and Tatum 1973). Practical use of auxotrophic markers, however, awaited the demonstration of transformation of protoplasts of a leucine-requiring Saccharomyces strain with the DNA from a leucine prototroph (Hinnen et al. 1978) and soon thereafter with the LEU2 gene on an autonomously replicating shuttle plasmid (Beggs 1978). Among the most commonly used selectable markers are complementation of nutrient requirements, such as histidine (Case et al. 1979) and pyrimidines (Ballance et al. 1983). Other auxotrophies have been employed, such as tryptophan (Yelton et al. 1984), adenine (Kurtz et al. 1986), quinic acid (Case 1982), and nitrate reductase (Campbell et al. 1989).

15.4.2 Drug Resistance

Because complementation of auxotrophic strains has specific problems, the use of dominant markers such as drug resistance is highly desirable. For fungi this is complicated by the relatively few drugs available as drug/selectable marker systems. The most commonly used drug for selection in filamentous fungi is hygromycin, and many vectors are derived from the pAN7-1 plasmid first used to confer hygromycin resistance in *Aspergillus* (Punt et al. 1987). Other markers include bialaphos resistance (Avalos et al. 1989) and then less commonly used markers like sulfonylurea (Sweigard et al. 1997), phleomycin (Mattern et al. 1988), and nourseothricin (Kück and Hoff 2006).

15.4.3 Recyclable Markers

By careful expression of splicing sequences and the genes for their recognition, some markers can be evicted once transformation has been accomplished. These commonly use the cre-lox system and require induction of the genes for splicing. The end product is a transformed strain that no longer carries the selectable marker. These strains can then be transformed with the same marker increasing the value of such systems (Krappmann et al. 2005). In a similar vein, the FLP/FRT technique has also been adapted from *Saccharomyces cerevisiae* to recycle markers in *P. chrysogenum*, and other filamentous fungi, by codon optimization of the recombinase gene (KOPKE et al. 2010).

While not in the same category as markers that integrate into the genome and require subsequent excision, autonomously replicating plasmids are important for manipulation of some species. The AMA series of plasmids (Osherov et al. 2000) offer the benefit of autonomous replication for *Aspergillus* and have been widely used both as a genome library and as individual vectors for other uses. While there was some suggestion that autonomous replication could be observed in *Neurospora* (Grant et al. 1984), this has never been used to develop vectors for gene manipulation.

15.4.4 Novel Methods

Complementation of temperature-sensitive (TS) mutations was used as a means to identify the gene carrying the TS lesion. It was not until identification of the ribosomal S9 protein that this was proposed as a deliberate selectable marker. Subsequently this has been used to disrupt the *albino-1* gene in Neurospora in a strain carrying the TS allele. Complementation by the *Magnaporthe* ribosomal S9

gene allowed targeting without interference by homology with the native *Neurospora* locus (Wiest et al. 2012c).

Plasmids are naturally found in mitochondria of several fungi (Griffiths 1995), and while these suggest that it should offer a route for manipulation of fungal physiology, this has not proven to be useful in practice.

15.5 Plasmids/Strains

For fungi where dominant selectable markers are available, they are widely used, and a series of vectors based on the same genes are often available for use across multiple target species. With the demonstration that targeting was enhanced in strains deficient in nonhomologous end joining, many such strains were developed to allow modern gene targeting. Among these strains are representatives from most of the commonly used model species (Table 15.2).

15.5.1 Neurospora

Most strains of *Neurospora* are sensitive to hygromycin and so are readily transformed with the most commonly available vectors. Additional strains are used for special purpose transformation including auxotrophic strains, temperature-sensitive strains, and strains defective in nonhomologous end joining (Table 15.2). Among the auxotrophic complementation systems, the most widely used was the *his-3* complementation which allowed targeting to the *his-3* locus in *Neurospora*. This robust system generated significant numbers of homologous integrants, but because of the variability at *his-3* among laboratory strains (Yeadon et al. 1998), most researchers use strains carrying the 1-234-723 allele of *his-3* (Margolin et al. 1997).

15.5.2 Aspergillus

Some species of *Aspergillus* are naturally resistant to hygromycin, and so complementation of auxotrophies is the most common method for selecting for transformants. The most commonly used marker is pyrG (Oakley et al. 1987a), and several vectors exist for selecting transformants in the most commonly studied species of Aspergillus (Table 15.3). Similarly, complementation of mutations at the riboB locus (Oakley et al. 1987b) has been used for selecting transformants in *A. nidulans* and *A. fumigatus* (Nayak et al. 2006). One of the commonly used transformation systems for *Aspergillus* uses the *amdS* gene on the plasmid p3SR2 (Tilburn et al. 1983) to confer the ability to grow on acetamide as the sole nitrogen

Table 15.2 Strains engineered for targeted gene disruption

FGSC #	Species	Characteristics	Reference
8071	*N. crassa*	am target strain TEC39	Cambareri and Kinsey (1994)
8072	*N. crassa*	am target strain TEC41	Cambareri and Kinsey (1994)
9538	*N. crassa*	mus-51 delta::Bar; his-3	Ishibashi et al. (2006)
9539	*N. crassa*	mus-52 delta::Bar; his-3	Ishibashi et al. (2006)
9567	*N. crassa*	mus-52::Hyg^r	Ishibashi et al. (2006)
9568	*N. crassa*	mus-52::Hyg^r	Ishibashi et al. (2006)
9595	*N. crassa*	mus-51::Hyg^r	Ishibashi et al. (2006)
9717	*N. crassa*	delta mus-51::bar+; his-3	Ishibashi et al. (2006)
9719	*N. crassa*	delta mus-52::bar+	Ishibashi et al. (2006)
9720	*N. crassa*	delta mus-52::bar+; his-3	Ishibashi et al. (2006)
9718	*N. crassa*	delta mus-51::bar+	Ishibashi et al. (2006)
20277	*N. crassa*	NCU08290.2 (mus-51)	Colot et al. (2006)
20278	*N. crassa*	NCU08290.2 (mus-51)	Colot et al. (2006)
10216	*N. crassa*	un-16, mus-52	McCluskey et al. (2007)
10217	*N. crassa*	un-16, mus-52	McCluskey et al. (2007)
10218	*N. crassa*	un-16, mus-51	McCluskey et al. (2007)
10219	*N. crassa*	un-16, mus-51	McCluskey et al. (2007)
A1421	*A. flavus*	CA14 deltaKu70 delta PyrG	Chang et al. (2009)
A1181	*A. niger*	Δ kusA pyrG-	Meyer et al. (2007)
A1279	*A. niger*	KusA::amdS; pyrG	Carvalho et al. (2010)
A1180	*A. niger*	Delta kusA	Meyer et al. (2007)
A1515	*A. niger*	pyrG-, KusA::AfpyrG	Chiang et al. (2011)
A1182	*A. niger*	Delta kusA::AmdS	Meyer et al. (2007)
A1179	*A. niger*	Delta kusA pyrG-	Meyer et al. (2007)
A1421	*A. flavus*	CA14 deltaKu70 delta PyrG	Chang et al. (2009)
A1280	*A. fumigatus*	akuA::loxP	Hartmann et al. (2010)
A1160	*A. fumigatus*	DeltaKU80 pyrG-	Krappmann et al. (2006), Krappmann (2006)
A1159	*A. fumigatus*	akuA::loxP	Krappmann et al. (2006), Krappmann (2006)
A1158	*A. fumigatus*	akuA::loxP-hygro^R/tk	Krappmann et al. (2006), Krappmann (2006)
A1157	*A. fumigatus*	akuA::ptrA	Krappmann et al. (2006), Krappmann (2006)
A1151	*A. fumigatus*	pyrG^AF::Delta KU80	Krappmann et al. (2006), Krappmann (2006)
10386	*M. grisea*	P1.2-deltaKU80	Villalba et al. (2008)
10385	*M. grisea*	Guy11-deltaKU80	Villalba et al. (2008)
A1190	*A. parasiticus*	ordA, Ku70	Chang (2008)
A1243	*A. parasiticus*	Delta ku70	Ehrlich et al. (2008)
A1244	*A. parasiticus*	Delta ku70 Delta pyrG	Chang et al. (2009)

Table 15.3 Selectable markers and plasmids for transforming filamentous fungi

Selection	Marker	Plasmids	Reference
Dominant selection			
Benomyl	BmlR/ben	pBT6, pBenA3	Orbach et al. (1986), Jung et al. (1992)
Hygromycin	Hph/ HygR	pES20, etc.	Staben et al. (1989)
Bialaphos	bar	pBARKS1, etc.	Pall and Brunelli (1993)
Phleomycin	phleo	pBC-phleo	Silar (1995)
Sulfonylurea	sur	pCB1528, etc.	Sweigard et al. (1997)
Nourseothricin	nat1	pD-Nat1	Kuck and Hoff (2006)
Recessive selection			
Histidine	*his-3*	pNH60, pRAUW122, pJHAM002	Legerton and Yanofsky (1985), Aramayo and Metzenberg (1996), Lee et al. (2003)
Acetamide	*amd-S*	p2SR2	Wernars et al. (1985)
Purine	*pyrG*	ppyrG, pPL6, Anep2, etc.	Oakley et al. (1987a), Storms et al. (2005)
Pyridoxine	*pyroA*	pTN1, pFB6	Nayak et al. (2006)
Inositol	*inl*	pINL, pOKE01, pRATT19	Akins and Lambowitz (1985)
Riboflavin	*riboB*	pLO1, pPL1	Oakley et al. (1987b)
TS lethal	*un-16*	pUN16-6	McCluskey et al. (2007)

source. This plasmid is one of the most highly distributed (Table 15.4) and has been used for the transformation of a variety of fungi including *A. niger* (Kelly and Hynes 1985), *Cochliobolus* (Turgeon et al. 1985), *Penicillium* (Beri and Turner 1987), and *Trichoderma* (Rahman et al. 2009, p. 6942.

15.5.3 Other Fungi

While not all of the tools developed for use in *Neurospora* or *Aspergillus* are directly applicable to use in other research systems, many of the vectors used in these systems can be engineered to function in unrelated systems (Meyer 2008). Similarly, many of the approaches used in *Neurospora* and *Aspergillus* are employed to generate specific characteristics, such as chlorate resistance which is useful for forcing anastomosis (Bowden and Leslie 1992) or for transformation directly (Daboussi et al. 1989).

Finally, interspecific transfer of genes is especially useful when one is doing gene targeting. For example, the *Aspergillus nidulans riboB* gene is useful for targeting in *A. fumigatus* (Nayak et al. 2006), and the *Magnaporthe* ribosomal S9 gene can be used to transform *un-16* TS-lethal mutant strains of *Neurospora* without interfering with targeting [e.g., to the *al-1* locus (Wiest et al. 2012c)].

Table 15.4 Most commonly requested plasmids from the FGSC collection from January 2000 to September 2012

Plasmid name	Number of distributions	Reference
pSilent-1	114	Nakayashiki et al. (2005)
pBC-phleo	86	Silar (1995)
pCSN44	78	Staben et al. (1989)
pBARGPE1	71	Pall and Brunelli (1993)
pMF272	67	Freitag et al. (2004)
pRS426	63	Christianson et al. (1992)
gGFP	56	Maor et al. (1998)
pCB1003	56	Caroll et al. (1994)
pCB1004	52	Caroll et al. (1994)
pPK2	48	Covert et al. (2001)
pCSN43	46	Staben et al. (1989)
pBARMTE1	42	Pall and Brunelli (1993)
p3SR2	42	Wernars et al. (1985)
pBC-hygro	41	Silar (1995)
pMYX10	37	Campbell et al. (1994)
pMYX2	35	Campbell et al. (1994)
pMT-mRFP1	35	Toews et al. (2004)
pBARKS1	34	Pall and Brunelli (1993)
ppyrG	34	Oakley et al. (1987a)
pMT-BFP	34	Toews et al. (2004)
pMF280	32	Freitag et al. (2004)
pFNO3	32	Yang et al. (2004)
pAO81	30	Yang et al. (2004)
pRG3-AMA1-NotI	29	Liu et al. (2004)
pMF334	29	Freitag and Selker (2005)
pXDRFP4	28	Yang et al. (2004)
pMF309	28	Freitag et al. (2004)
cosmid An26	28	Taylor and Borgmann (1996)
pMT-sGFP	27	Toews et al. (2004)
pSD1	26	Nguyen et al. (2008)
pD-Nat1	25	Kuck and Hoff (2006)
pCB1532	24	Sweigard et al. (1997)
pMOcosX	24	Orbach (1994)
pMF332	24	Freitag and Selker (2005)
pME2891	22	Krappmann et al. (2005)
pMF331	22	Freitag and Selker (2005)
p500	22	Vogt et al. (2005)
pMG2254	22	Gerami-Nejad et al. (2009)
pCCG::N-GFP	21	Honda and Selker (2009)
pA-HYG OSCAR	21	Paz et al. (2011)
pBARGEM7-2	21	Pall and Brunelli (1993)
pCCG::C-Gly::HAT::FLAG	21	Honda and Selker (2009)
pBT6	21	Orbach et al. (1986)
pYFP	20	Bardiya et al. (2008)
pOSCAR	20	Paz et al. (2011)

15.6 Fluorescent Proteins

15.6.1 Neurospora

GFP constructs were available for other organisms (Niedenthal et al. 1996; Zeilinger 1999, p. 68) before Freitag developed a codon-optimized version of GFP for *Neurospora* (Freitag et al. 2004). After this marker was introduced into the research community, numerous related fluorescent-tagged proteins were developed for use in *Neurospora* (Table 15.5). These include a split yellow fluorescent protein marker useful for the study of protein interactions (Hammond et al. 2011), as well as many fluorescent proteins targeted for specific organelles (Bowman et al. 2009). In addition to the fluorescent proteins described above, several vectors are available which will allow visualization of protein levels or location. The *lux* gene, derived from the firefly luciferase gene, has been engineered for use in *Neurospora* (Morgan et al. 2003) and ultimately employed as a signal of the levels of expression of the product of the *frq* locus (Gooch et al. 2008).

15.6.2 Aspergillus

With myriad fluorescent-tagged proteins available on convenient plasmids (Table 15.5), the growth in distribution of these plasmids reflects their value to the community (Table 15.4). The series developed in the laboratory of R. Fischer (Toews et al. 2004) includes green, red, and blue fluorescent proteins; these are all available from the FGSC and have been widely used in developing tools within the genus *Aspergillus* (Lubertozzi and Keasling 2009), as well as for cell biology studies in *Aspergillus* (Arratia-Quijada et al. 2012) and in unrelated fungi (Helber and Requena 2008). This reiterates the situation with selectable markers and tags; many of the tools developed for *Aspergillus* find direct utility in other systems underscoring the value of tool development in model systems.

15.6.3 Other Fungi

The most commonly used fluorescent protein used in a variety of systems is encoded by the gGFP vector developed by A. Sharon (Maor et al. 1998). This vector includes a hygromycin resistance cassette driven by the *A. nidulans* glyceraldehyde 3-phosphate dehydrogenase promoter and the terminator from the *A. nidulans trpC* gene. The GFP gene is also driven by the *gpd* promoter and uses the GFP gene and terminator from the plasmid pHSP70-SG (Spellig et al. 1996) which was originally generated for use in *U. maydis*. The gGFP plasmid was optimized for use in *Cochliobolus*, but has been requested for use in a broad variety of fungal systems including *Colletotrichum* (Horowitz 2002), *Verticillium* (Eynck et al. 2007), and others (Lorang et al. 2001).

Table 15.5 Plasmids carrying visualization tags

Name	Tag	Organism	Reference
pFNO3	GFP	Aspergillus	Yang et al. (2004)
pHL86	GA5-chRFP, riboB	Aspergillus	Liu et al. (2009)
pHL85	chRFP, pyroA	Aspergillus	Liu et al. (2009)
pHL84	GA5-GFP, pyroA	Aspergillus	Liu et al. (2009)
pHL83	GA5-GFP, loxP, pyrG	Aspergillus	Liu et al. (2009)
pHL82	GA5-GFP, riboB	Aspergillus	Liu et al. (2009)
pXDRFP4	RFP	Aspergillus	Yang et al. (2004)
pSK800	mRFP1	Aspergillus	Toews et al. (2004)
pSK494	GFP2-5	Aspergillus	Szewczyk and Krappmann (2010)
pSK495	yfp	Aspergillus	Szewczyk and Krappmann (2010)
pSK496	mCherry	Aspergillus	Szewczyk and Krappmann (2010)
pJH19	DsRedT4	Aspergillus	Toews et al. (2004)
pRF281	GFP	Aspergillus	Toews et al. (2004)
pDV2	sGFP	Aspergillus	Toews et al. (2004)
pRS54	GFP	Aspergillus	Suelmann and Fischer (2000)
pSK700	DsRedT4	Aspergillus	Toews et al. (2004)
pMT-sGFP	sGFP	Aspergillus	Toews et al. (2004)
pMT-BFP	BFP	Aspergillus	Toews et al. (2004)
pMT-mRFP1	mRFP1	Aspergillus	Toews et al. (2004)
pPND1	mRFP1	Aspergillus	Rischitor et al. (2004)
pRF280	GFP	Aspergillus	Toews et al. (2004)
pOT-eGFP	eGFP	Botrytis	Patel et al. (2008)
pOT-LUC	Luc	Botrytis	Patel et al. (2008)
pMG2082	GFP-URA3-GFP	Candida	Gerami-Nejad et al. (2009)
pMG1958	Ppck1-GFP	Candida	Gerami-Nejad et al. (2004)
pMG1892	pgal-GFP	Candida	Gerami-Nejad et al. (2004)
pMG1886	pmet3-GFP	Candida	Gerami-Nejad et al. (2004)
pMG1726	CFP-URA3	Candida	Gerami-Nejad et al. (2001)
pMG1648	YFP-URA3	Candida	Gerami-Nejad et al. (2001)
pMG1602	GFP-URA3	Candida	Gerami-Nejad et al. (2001)
pMG1801	CFP-His1	Candida	Gerami-Nejad et al. (2001)
pMG2169	RFP-URA3	Candida	Gerami-Nejad et al. (2009)
pMG1646	GFP-His1	Candida	Gerami-Nejad et al. (2001)
pMG2254	M-cherry-TADH-URA3	Candida	Gerami-Nejad et al. (2009)
pMG1656	YFP-His1	Candida	Gerami-Nejad et al. (2001)
pCAMDsRED	DsRed-Express	Leptosphaeria	Eckert et al. (2005)
gGFP	GFP	Many	Maor et al. (1998)
pVG101	ccg2p-o-luc-I	Neurospora	Gooch et al. (2008)
pHAN1	sgfp, HA	Neurospora	Kawabata and Inoue (2007)
pMF334	RFP	Neurospora	Freitag and Selker (2005)
pMF332	RFP	Neurospora	Freitag and Selker (2005)
pMF331	RFP	Neurospora	Freitag and Selker (2005)

(continued)

Table 15.5 (continued)

Name	Tag	Organism	Reference
pAL3-Lifeact	TagRFP	Neurospora	Berepiki et al. (2010)
pAL4-Lifeact	nat1 TagRFP	Neurospora	Lichius and Read (2010)
pAL5-Lifeact	TagRFP-T	Neurospora	Lichius and Read (2010)
pAL6-Lifeact	TagRFP	Neurospora	Lichius and Read (2010)
pAL12-Lifeact	TagRFP	Neurospora	Lichius and Read (2010)
pYFPN	YFP	Neurospora	Bardiya et al. (2008)
pTH1124.1	YFPC-	Neurospora	Hammond et al. (2011)
pTH1123.1	YFPN-	Neurospora	Hammond et al. (2011)
pTH1117.12	GFP-	Neurospora	Hammond et al. (2011)
pTH1112.8	-YFPN	Neurospora	Hammond et al. (2011)
pTH1111.1	-RFP	Neurospora	Hammond et al. (2011)
pTH1108.2	-YFPC	Neurospora	Hammond et al. (2011)
pMF309	Bml-GFP	Neurospora	Freitag et al. (2004)
pMF280	hH1-GFP	Neurospora	Freitag et al. (2004)
pMF272	GFP	Neurospora	Freitag et al. (2004)
pLUC6 delta BS	lux	Neurospora	Morgan et al. (2003)
pAL10-Lifeact	TagRFP	Neurospora	Lichius and Read (2010)
pRFP-vps-52	RFP	Neurospora	Bowman et al. (2009)
pRFP-cax	RFP	Neurospora	Bowman et al. (2009)
pRFP-nca-1	RFP	Neurospora	Bowman et al. (2009)
pnca-2-GFP	GFP	Neurospora	Bowman et al. (2009)
pRFP-nca-2	RFP	Neurospora	Bowman et al. (2009)
pnca-3-GFP	GFP	Neurospora	Bowman et al. (2009)
pgrp-GFP	GFP	Neurospora	Bowman et al. (2009)
pTH1067.9	GFP	Neurospora	Hammond et al. (2011)
pRFP-grp	RFP	Neurospora	Bowman et al. (2009)
pdpm-GFP	GFP	Neurospora	Bowman et al. (2009)
pVG110	frqp-o-luc-I	Neurospora	Gooch et al. (2008)
pvps-52-GFP	GFP	Neurospora	Bowman et al. (2009)
pYFP	YFP	Neurospora	Bardiya et al. (2008)
pRFP-vam-3	RFP	Neurospora	Bowman et al. (2009)
pRFP-vma-1	RFP	Neurospora	Bowman et al. (2009)
parg-4-GFP	GFP	Neurospora	Bowman et al. (2009)
pAL1	sGFP	Neurospora	Berepiki et al. (2010)
pAL2-Lifeact	tdTomato	Neurospora	Lichius and Read (2010)
pGFP::hph::loxP	GFP	Neurospora	Honda and Selker (2009)
pCCG::N-GFP	GFP	Neurospora	Honda and Selker (2009)
pCCG::C-Gly::GFP	GFP	Neurospora	Honda and Selker (2009)
pYFPC	YFP	Neurospora	Bardiya et al. (2008)
pnca-1-GFP	GFP	Neurospora	Bowman et al. (2009)
pRFP-dpm	RFP	Neurospora	Bowman et al. (2009)

15.7 Tags

15.7.1 Visualization

Several proteins have been used as target for antibodies when linked to a polypeptide under study. Among them, the GFP protein was localized by immunological reaction using a rabbit anti-GFP antibody (Gordon et al. 2000).

15.7.2 Purification

Numerous protein tags have been developed to facilitate protein purification, and many of these are available in public collections. For example, the FLAG (Honda and Selker 2009), S-TAG (Yang et al. 2004), and HA (Toews et al. 2004) tags are present among many of the most often distributed plasmids from the FGSC collection (Table 15.4). The application of these tags allows rapid protein purification and is having an impact on fungal proteomics (Liu et al. 2009) and on understanding of the fungal protein interactome (Wang et al. 2011). If the distribution of these plasmids (Table 15.4) is a predictor of the impact that they may have, we can expect additional examples of their application to understanding the role of specific proteins in numerous important questions of fungal development, ultrastructure, and environmental interactions.

15.8 Conclusion

15.8.1 Impact

Since 2000, the FGSC has distributed over 3,545 individual plasmid samples. Among these, the most popular plasmids are those for transformation and manipulation of model filamentous fungi (Table 15.4). Similarly, strains for targeted transformation have had a huge impact, and the combined availability of these strains and the plasmids for targeted transformation have allowed the development of a number of systematic and targeted gene deletion programs. This is arguably the biggest advancement in research in filamentous fungi for many decades, and it allows evaluation of the 80–90 % of genes that are not discoverable in traditional mutagenesis programs. In *N. crassa*, a set of mutant strains was generated where nearly every gene has been deleted in a strain engineered to be defective in nonhomologous end joining (Colot et al. 2006). This set was arrayed at the FGSC and has been distributed as arrayed strains to laboratories in the USA, Europe, Asia, and South America (Wiest et al. 2012b). Similar sets have been prepared, although

not in a systematic manner, for *Candida albicans*, *Cryptococcus neoformans*, and, of course, for yeast. There is significant interest in generating similar resources for plant-pathogenic fungi, and a set of 48,000 tagged-integrant strains of *Magnaporthe grisea* were developed and deposited at the FGSC (Betts et al. 2007). Regrettably, the requirement is that every lab that wants to work with these strains receives a permit from the USDA Biotechnology Regulatory Service, which has limited the impact of these materials. Similar permits would be required for genetically engineered strains of any plant pathogen, and until this is resolved, it is unlikely that a systematic gene deletion resource will be made available for these important organisms.

15.8.2 Future Prospects

Most of the molecular tools used for manipulation of filamentous fungi are available from the Fungal Genetics Stock Center. In 2012, the FGSC entered into negotiations with Addgene (http://www.addgene.com), a nonprofit plasmid repository, to deposit many of the most highly requested plasmids. This is both good practice for an active collection (second-site backup is part of the best practice guidelines for microbial germplasm repositories) and increases the impact of each plasmid, by increasing their visibility to potentially new customers.

Additionally, the development of novel organism systems for applications such as biomass deconstruction, pharmaceutical production, biocontrol, or the production of food and fiber will mean that new tools for manipulation and engineering of these fungi will be developed. The sharing of these materials via active well-curated collections assures that these materials will have the greatest impact (Furman and Stern 2011) and that they will be available long after their primary use has been accomplished.

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B.A. Horwitz et al. (eds.), *Genomics of Soil- and Plant-Associated Fungi*, Soil Biology 36, DOI 10.1007/978-3-642-39339-6,

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